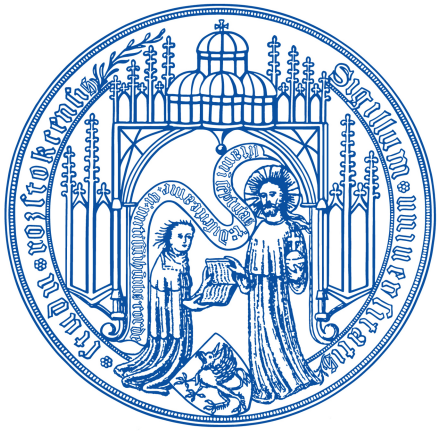
Unraveling the regulatory mechanisms controlling the biosynthesis and emission of the volatile organic compound 'sodorifen' produced by *Serratia plymuthica* 4Rx13

A thesis to acquire the academic degree of
***doctor rerum naturalium* (Dr. rer. nat.)**
at the Faculty of Mathematics and Natural Sciences,
Institute of Biological Sciences,
University of Rostock



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March 2018

urn:nbn:de:gbv:28-diss2018-0157-0

To my mother and Daniel

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Year of submission	2018
Year of defense	2018

Abstract

It is well established that bacteria produce a wealth of volatile organic compounds (VOCs) which, for example, can serve as antimicrobials or natural biofertilizers. However, to date research on inter- as well as intraspecific activities of microbial VOCs still remains in its infancy.

Likewise, the rhizobacterium *Serratia plymuthica* 4Rx13 represents a rich source of VOCs by emitting up to 100 different compounds with the unique compound 'sodorifen' accounting for about 45 % of the total volatile bouquet. It was found to be a polymethylated bicyclic sesquiterpene whose biosynthesis depends on a four-gene cluster (sodorifen cluster) encoding a terpene cyclase, methyltransferase, DOXP synthase and IPP isomerase.

This PhD thesis is supposed to shed light on the biological function of the unusual volatile sodorifen by investigation of regulatory mechanisms underlying its biosynthesis and emission. Interestingly, the genetically closely related strain *S. ply.* AS9 also contains the sodorifen cluster but does not emit sodorifen. Expression analyses, using RT-PCRs and Northern blots, could prove that the sodorifen cluster in AS9 is silent, indicating transcriptional regulation of sodorifen emission.

To assess potential transcriptional regulators, the upstream region of the sodorifen cluster was analyzed. Thereby, a σ^S dependent promoter was identified which is specifically responsible for the expression of genes during stationary phase growth. Moreover, a σ^S-negative mutant exhibited a sodorifen-negative phenotype. Additionally, sodorifen emission was found to vary in response to application of different carbon sources. Especially interesting was the observation that sodorifen emission is repressed during presence of glucose as the sole carbon source. Using insertional deletion mutagenesis the global regulatory mechanism of carbon catabolite repression was found to be responsible for transcriptional repression due to glucose application. Furthermore, sodorifen cluster transcription was found to be increased in response to cell density by activation of a LuxI/R-like quorum sensing system.

In conclusion, this work presents substantial knowledge about the different regulatory levels controlling sodorifen emission. Moreover, the necessity of the σ^S factor for sodorifen cluster expression leads to the speculation that sodorifen might enable its producers to cope with certain stress conditions and thus, provide a survival benefit to them.

Zusammenfassung

Es ist allgemein bekannt, dass Bakterien ein großes Spektrum volatiler organischer Verbindungen (engl. *volatile organic compounds*, VOCs) produzieren, die unter anderem als Antibiotika oder natürliche Düngemittel Verwendung finden. Allerdings befindet sich die Forschung bezüglich der inter- und intraspezifischen Wirkungsweisen mikrobieller VOCs derzeit noch in ihren Anfängen.

So besitzt z.B. das Rhizobakterium *Serratia plymuthica* 4Rx13 ein sehr reiches Volatilen-Spektrum mit einer bislang unbekannten Hauptkomponente, 'Sodorifen', die bis zu 45 % des gesamten Bouquets ausmachte. Dabei handelt es sich um ein polymethyliertes, bizyklisches Sesquiterpen, dessen Biosynthese von der Expression eines Vier-Gen-Clusters (Sodorifen-Cluster), bestehend aus einer Terpen-Cyclase, Methyltransferase, DOXP-Synthase und IPP Isomerase abhängig ist.

Ziel dieser Dissertation sollte es sein, die biologische Funktion der ungewöhnlichen volatilen Verbindung Sodorifen, durch Untersuchung der zugrundeliegenden regulatorischen Mechanismen der Sodorifen-Biosynthesegene, aufzuklären. Interessanterweise, besitzt der genetisch eng verwandte Stamm *S. ply.* AS9 ebenfalls das Sodorifen-Cluster, emittiert jedoch kein Sodorifen. Dies konnte, mittels RT-PCR- und Northern Blot-Analysen, auf eine fehlende Expression des Biosynthese-Clusters zurückgeführt werden, was auf eine transkriptionelle Regulation der Sodorifen-Emission hinwies. Zudem konnten Untersuchungen der Promotersequenz erstmals zeigen, dass die Transkription des Sodorifen-Clusters vom σ^S-Faktor initiiert wird, da durch Ausschalten dieses Faktors ein Sodorifen-negativer Phänotyp hervorgerufen wurde. Eine weitere regulatorische Ebene der Sodorifen-Emission zeigte sich in Abhängigkeit der verfügbaren Kohlenstoffquelle, wobei besonders die reprimierende Wirkung von Glucose auffiel. Durch gezielte Mutagenese konnte nachgewiesen werden, dass der globale Regulationsmechanismus der Kohlenstoff-Katabolit-Repression für die verringerte Sodorifen-Clusterexpression in Gegenwart von Glucose verantwortlich ist. Weiterhin konnte eine positive, Zelldichte-abhängige Regulation der Sodorifen-Emission demonstriert werden, welche durch Aktivierung eines, zu LuxI/R homologen, Quorum Sensing Systems erreicht wurde.

Schlussendlich, konnte diese Arbeit ein umfassendes Bild, bezüglich der Regulation der Sodorifen-Emission in *S. ply.* 4Rx13 liefern. Darüber hinaus lässt vor allem die Tatsache, dass die Sodorifen-Cluster Expression σ^S-abhängig erfolgt, die Schlussfolgerung zu, dass Sodorifen eine potenzielle Rolle in der Stressbewältigung des produzierenden Organismus spielen und somit einen überlebenswichtigen Vorteil in der Natur darstellen könnte.

Contents

Abbreviations

Abbreviation	meaning
%	percent
&	and
°C	degree Celsius
µ	micro
ad	fill up to
Amp	ampicillin
bp	base pairs
BLAST	Basic Local Alignment Search Tool
cAMP	3',5'-cyclic adenosine monophosphate
CCR	carbon catabolite repression
CDP star	Disodium 2-chloro-5-(4-methoxyspiro 1,2-dioxetane-3,2'-(5'-chloro) tricyclo[3.3.1.1^{3,7}] decan-4-yl)-1-phenyl phosphate
CFU	colony forming units
CRE	catabolite responsive element
CRP	cAMP receptor protein
ddH_2O	double distilled water
DE	Germany
DEPC	diethyl pyrocarbonate
DIG	digoxigenin
DMM	Davis-Mignoli minimal medium
DNA	deoxyribonucleic acid
DOXP	1-Deoxy-D-xylulose 5-phosphate
DXS	1-Deoxy-D-xylulose 5-phosphate synthase
E. coli	*Escherichia coli*
e.g.	for example
et al.	et alii
F	Farad
FPP	farnesylpyrophosphate
g	gram
x g	times Earth's gravitational force
G3P	glyceraldehyde 3-phosphate
GC-MS	gas chromatography-mass spectrometry

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Abbreviation	**meaning**
glc	glucose
h	hour(s)
H_2O	water
i.e.	that means
k	kilo
kb	kilobase
Km	kanamycin
l	litre
LB	Luria-Bertani medium
m	milli
M	Mega
m/z	mass-to-charge ratio
met	methionine
$MgCl_2$	magnesium chloride
min	minute(s)
MM	minimal medium
mRNA	messenger ribonucleic acid
mVOC	microbial volatile organic compound
NaCl	sodium chloride
NPKM	nucleotide activity per kilobase of exon model per million mapped reads
Ω	ohm
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
pH	*potenia hydrogenii*
Pyr	pyruvate
QS	quorum sensing
RNA	ribonucleic acid
RNAP	ribonucleic acid polymerase
rpm	revolutions per minute
RT	reverse transcriptase
s	second(s)
S. ply.	*Serratia plymuthica*
SDS	sodium dodecyl sulfate

continued on next page

Abbreviation	**meaning**
SPME	solid phase micro extraction
succ	succinate
Tet	tetracyclin
USA	United States of America
V	volt
VOC	volatile organic compound
vol	volumes

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1 Introduction

1.1 Soil microbes

According to the Soil Science Society of America (2018), the soil is defined as the layer(s) of generally loose mineral and/or organic material that are affected by physical, chemical, and/or biological processes at or near the planetary surface, and usually holds liquids, gases and biota and supports plants. In general, soil is considered to be a complex habitat possessing the highest microbial diversity on earth and is characterized by its highly heterogeneous but still nutrient-limited environment (Tyc *et al.*, 2017b). Depending on the soil type, the number of prokaryotes present in this biosphere is estimated to vary from 10^{10} to 10^{11} per gram of soil (Horner-Devine *et al.*, 2003) and comprised of 6.000-50.000 different bacterial species (Curtis *et al.*, 2002).

The distribution and reproduction of microorganisms in the soil can vary significantly and is influenced by diverse factors including root growth and environment (Bürgmann *et al.*, 2005). Accordingly, bacterial density was found to be increased 10-1.000 fold in the rhizosphere, i.e. the part of the soil that is in direct contact to the plant roots, in comparison to bulk soil (Lugtenberg and Kamilova, 2009). This is due to the fact that plant roots are capable of secreting 10-30 % of their photosynthates as root exudates and thus, provide potential nutrients for microorganisms (Bowen and Rovira, 1999; Shaw *et al.*, 2006; Haichar *et al.*, 2008). Plant root exudates consist of a complex mixture of organic acid anions, phytosiderophores, sugars, vitamins, amino acids, purines, nucleosides, inorganic ions (e.g. HCO_3^-, OH^-, H^+) (Prescott and Dunn, 1949), gaseous molecules (CO_2, H_2), enzymes and root border cells which have major direct or indirect effects on the acquisition of mineral nutrients required for plant growth (Dakora and Phillips, 2002; Dotaniya and Meena, 2015). Furthermore, rhizosphere soil has been proposed to have a higher affinity for water due to exudates secreted by the plant roots (Whiteley, 1989; Young, 1995). As a result, the rhizosphere represents a hotspot of microbial proliferation and activity which is also referred to as the "rhizosphere effect" (Raaijmakers *et al.*, 2009).

High nutrient abundances in the rhizosphere not only favour colonization by bacteria but also increase the appearance of other organisms, e.g. fungi, protozoans and nematodes. Consequently, positive as well as negative interplays take place between different organisms. Negative interactions occur via competition, parasitism or pathogenesis caused by bacteria, fungi or herbivorous invertebrates whereas positive interactions include mutualism and symbiosis of plants with epiphytes, my-

corrhizal fungi and also rhizobacteria (reviewed in Bais *et al.* 2006).

1.1.1 Plant growth-promoting rhizobacteria (PGPR)

As indicated above, rhizobacteria can positively affect plant health while taking advantage of their secreted root exudates. Accordingly, they are often referred to as plant growth-promoting rhizobacteria (PGPR) and increase plant fitness by various mechanisms. On the one hand, PGPR can enhance nutrient availability for plants by nitrogen fixation, phosphate solubilization or phytosiderophore production (Richardson *et al.*, 2009). On the other hand, the production of plant hormones or other compounds interfering with plant hormone production can improve plant fitness, e.g. by enhancing the development of root hairs (Combes-Meynet *et al.*, 2011; Chamam *et al.*, 2013) and thereby the uptake of water and minerals (Vacheron *et al.*, 2013). Additionally, PGPR can influence plant health through inhibition of phytoparasites, either directly by antagonism and competition (Nielsen *et al.*, 2002) or indirectly by stimulation of plant defence mechanisms like the induced systemic resistance (ISR; Couillerot *et al.* 2009; Lugtenberg and Kamilova 2009; Vacheron *et al.* 2013).

So far, several bacterial species have been reported to enhance plant growth, among them are *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Alcaligenes*, *Arth-robacter*, *Enterobacter*, *Rhizobium*, *Burkholderia*, *Bacillus*, and *Serratia* species (Kloepper *et al.*, 1989; Okon and Labandera-Gonzalez, 1994; Glick, 1995; Joseph *et al.*, 2007).

Due to the negative impact of artificial fertilizers in agriculture, the use of PGPR as natural biofertilizers represents a promising approach for sustainable and ecological farming (Singh, 2013). Moreover, rhizobacteria can promote phytoremediation of heavy metal contaminated soil (Zhuang *et al.*, 2007; Shukla *et al.*, 2011; Saharan and Nehra, 2011; Tak *et al.*, 2013). For all of these reasons, research on the action of rhizobacteria is a high priority topic in science.

1.1.2 The genus *Serratia*

This study is especially concerned with bacteria belonging to the genus *Serratia*. Members of this genus belong to the class of γ-Proteobacteria and family of Enterobacteriaceae. Moreover, these Gram-negative bacteria are rod-shaped, peritrichously flagellated and possess a facultative anaerobic metabolism. They can be found ubiquitously in nature, colonizing diverse habitats like water, soil, plants, insects, vertebrates, and also humans (summarized in Grimont and Grimont 2006).

The type species, *Serratia marcescens*, was first described in 1819 and characterized in 1823 by Bizio after the appearance of red spots on polenta ("bloody

polenta") in the Italian province of Padua. Initially, *S. marcescens* was thought to be nonpathogenic. It was only after the Second World War when increasing evidence revealed the pathogenic potential of the organism. Today, *S. marcescens* is known to be capable of causing a wide range of opportunistic diseases (Thayer, 1966; Mahlen, 2011). Nevertheless, not all members of the genus *Serratia* have to be considered as pathogens.

In 2016, the genus *Serratia* was described to comprise 19 species. This includes, besides *Serratia marcescens*: 1) *Serratia aquatilis* (Kämpfer and Glaeser, 2016), 2) *Serratia entomophila* (Grimont *et al.*, 1988), 3) *Serratia ficaria* (Grimont *et al.*, 1979), 4) *Serratia fonticola* (Gavini *et al.*, 1979), 5) *Serratia glossinae* (Geiger *et al.*, 2010), 6) *Serratia grimesii* (Grimont *et al.*, 1982), 7) *Serratia liquefaciens* (Grimes and Hennerty, 1931; Bascomb *et al.*, 1971), 8) *Serratia myotis* (García-Fraile *et al.*, 2015), 9) *Serratia nematodiphila* (Zhang *et al.*, 2009), 10) *Serratia odorifera* (Grimont *et al.*, 1978), 11) *Serratia plymuthica* (Lehmann and Neumann, 1896; Breed *et al.*, 1948), 12) *Serratia proteamaculans* (Paine and Stansfield, 1919; Grimont *et al.*, 1978), 13) *Serratia quinivorans* (Grimont *et al.*, 1982; Ashelford *et al.*, 2002), 14) *Serratia rubidaea* (also referred to as *Serratia marinorubra*, Stapp 1940; ZoBell and Upham 1944; Ewing *et al.* 1973), 15) *Serratia symbiotica* (Sabri *et al.*, 2011), 16) *Serratia ureilytica* (Bhadra *et al.*, 2005), 17) *Serratia vespertilionis* (García-Fraile *et al.*, 2015) and most recently 18) *Serratia surfactantfaciens* (Su *et al.*, 2016).

In this study, the strain *S. plymuthica* 4Rx13 (formerly known as *S. odorifera 4Rx13, abbreviated S. ply. 4Rx13*) was of special interest. It was first isolated from the rhizosphere of oilseed rape (*Brassica napus* L.; Berg *et al.* 2002) and its genome, proteome as well as transcriptome have been extensively studied (Domik, 2012; Weise *et al.*, 2014; Domik *et al.*, 2016b). The genome of *S. ply.* 4Rx13 was found to be 5.4 Mb in size with a GC content of 56.09 % and consists of a bacterial chromosome (5.33 Mb) and an additional plasmid (ca. 75 kb) (Weise *et al.*, 2014).

1.2 Microbial secondary metabolites

Secondary metabolites are defined as small molecules which are not essential for survival but provide an advantage for the producing organism (Demain and Fang, 2000; Craney *et al.*, 2013). Bacteria start to produce these metabolites usually during the late growth phase (idiophase) upon nutrient depletion and thus, biosynthesis goes along with low growth rates (Demain, 1998; Ruiz *et al.*, 2010). The best studied and most pronounced microbial secondary metabolite producers are actinomycetes, which synthesize ca. 74 % of all described antibiotics (Miyadoh, 1993). Especially, members of the genus *Streptomyces* are known to synthesize a large number of

secondary metabolites, e.g. *Streptomyces hygroscopius* produces more than 180 different compounds (Zedan, 1993). Overall, microorganisms are estimated to be the source of up to 50.000 secondary metabolites (Fenical and Jensen, 1993; Berdy, 1995).

Microbially produced secondary metabolites include peptides, polyketides, carbohydrates, lipids, terpenoids, steroids, and alkaloids. They are synthesized from intermediates of the primary metabolism, e.g. acetyl-CoA, propionyl-CoA or intermediates of the shikimic acid pathway, the tricarboxylic acid cycle, and from amino acids (Demain and Fang, 2000; O'Brien and Wright, 2011).

Just like *Streptomyces* spp., also members of the genus *Serratia* are capable to produce several biologically relevant compounds. The probably most well-known is the secondary metabolite prodigiosin, a non-diffusible red pigment which was identified to be the cause of the aforementioned "bloody polenta" (Grimont and Grimont, 2006; Mahlen, 2011). It is produced by bacteria belonging to the species *S. marcescens*, *S. plymuthica* and *S. rubidaea* (Grimont and Grimont, 2006) by enzymatic condensation of 2-methyl-3-amylpyrrole (MAP) and 4-methoxy-2,2'-bipyrrole-5-carboxyaldehyde (MBC) (Williams and Qadri, 1980). According to the literature, prodigiosin develops antibacterial, antimalarial, antifungal and antiprotozoal activities (Williams and Qadri, 1980; Demain, 1995). Other well studied secondary metabolite produced by *Serratia* spp. are the broad spectrum β-lactam antibiotic carbapenem (1-carbapen-2-em-3-carboxylic acid; Williams and Qadri 1980; Parker *et al.* 1982) and the antifungal and antitumoral polyketide oocydin A (Srobel *et al.*, 1999; Matilla *et al.*, 2012).

Today, the discovery of new antimicrobial secondary metabolites is a high priority topic in research in order to combat the increasing number of multiresistant human pathogens. Due to advances in microbial genomics and bioinformatic annotation (Torrieri *et al.*, 2012; Wood *et al.*, 2012), a large number of gene clusters, encoding for enzymes synthesizing potentially new secondary metabolites could be identified which are not expressed under standard laboratory conditions (Zerikly and Challis, 2009; Winter *et al.*, 2011). Like this, underexplored or neglected organisms can be identified as alternative resources for new therapeutic agents and thus substantially contribute to pharmaceutical progress (Winter *et al.*, 2011).

1.2.1 Volatile organic compounds (VOCs)

Volatile organic compounds (VOCs) represent a large group of secondary metabolites that have been neglected for a long time in science. Characteristically, they are low molecular weight compounds (< 300 Da) with a high vapour pressure (0.01 kPa at 20 °C) a low boiling point and low polarity which facilitate their evaporation (Rowan,

2011). Consequently, they can serve as ideal infochemicals, acting over short and long distances as well as above- and belowground (Wheatley, 2002; van Dam *et al.*, 2010; Effmert *et al.*, 2012). VOCs are chemically diverse belonging to the groups of alkenes, alcohols, ketones, benzenoids, pyrazines, sulfides and terpenes (Schulz and Dickschat, 2007; Kanchiswamy *et al.*, 2015; Schmidt *et al.*, 2015).

Volatiles are known to be produced by all kinds of organisms, i.e. humans (Curran *et al.*, 2005), animals (Pfeiffer and Johnston, 1994; Schulz *et al.*, 2004), plants (Lund and Bohlmann, 2006; Kaiser, 2006), fungi (Schnürer *et al.*, 1999), and, most importantly, bacteria. Only recently, a database comprising all known microbial volatiles (mVOCs) has been published and is updated on a regular basis (Lemfack *et al.*, 2014, 2018). Until now, more than 2.000 discrete volatile organic compounds have been reported to be emitted by ca. 1.000 bacterial and fungal species (Lemfack *et al.*, 2018). Considering that 10^{16} microbial species are suspected to exist on earth, the lack of knowledge concerning volatile compounds becomes even more evident (Farré-Armengol *et al.*, 2016; Piechulla *et al.*, 2017).

In the past years, special efforts were put on the elucidation of biological functions of these special compounds. As a result, several beneficial as well as harmful effects of mVOCs on other organisms were identified (summarized in Wenke *et al.* 2010; Schmidt *et al.* 2015). Regarding plants, rhizobacterial volatiles have been found to increase plant fitness, e.g. directly by serving as nutrients (Meldau *et al.*, 2013), as well as indirectly by induction of the plants resistance to pathogens (Ryu *et al.*, 2004; D'Alessandro *et al.*, 2014; Wintermans *et al.*, 2016), inhibition of plant pathogens (Kai *et al.*, 2009; Garbeva *et al.*, 2014; de Vrieze *et al.*, 2015), or alteration of plant anatomy, physiology as well as hormone and biomass production (Wenke *et al.*, 2012; Bailly *et al.*, 2014; Piechulla *et al.*, 2017). Also fungi have been shown to react in response to bacterial volatiles. Like in plants, these interactions were found to be either positive or negative for the receiving fungal species (Vespermann *et al.*, 2007; Effmert *et al.*, 2012; Cordovez *et al.*, 2015; Ossowicki *et al.*, 2017). Nevertheless, volatiles are not only responsible for inter-kingdom communication but also elicit reactions in other bacterial species. Since the number of bacterial species is very high in the rhizosphere, competition for nutrients demands strategies to eliminate potential opponents. Respectively, studies by Tyc *et al.* (2017a) revealed that 2.5-bis(1-methylethyl)-pyrazine produced by the rhizobacterium *Paenibacillus* sp. AD87 inhibited growth of *Burkholderia* sp. AD24. Nevertheless, despite all of the aforementioned examples of volatile effects in nature, there is still a substantial lack of knowledge concerning microbial volatiles and their ecological function. Consequently, it will be of great interest to investigate volatile affairs in the future.

1.2.2 Sodorifen

The volatile spectrum of the rhizobacterium *Serratia plymuthica* 4Rx13 has been extensively studied in the past years. As a result, *S. ply.* 4Rx13 was found to emit more than 100 different volatile compounds, e.g. methanol, ethanol, methanethiol, dimethyl disulfide, dimethyl trisulfide, 2-phenylethanol, and other aromatic compounds as well as several ketones (Kai *et al.*, 2010; Weise *et al.*, 2014). Nevertheless, only ca. 10 % of the detected volatile compounds in *S. ply.* 4Rx13 could be identified during initial analyses (Kai *et al.*, 2010).

Interestingly, there was one very dominant substance found among the unidentified compounds, comprising up to 48 % of the total volatile bouquet. Furthermore, the mass spectrum of this, so far unknown, volatile revealed a molecular ion at *m/z* 218 and an unusual cluster of highly abundant fragment ions at *m/z* 136, 135, and 134 (**figure 1.1 B**, von Reuß *et al.* 2010). Using coupled GC/HRMS, the molecular formula was assigned to be $C_{16}H_{26}$. Additionally, the structure of this novel compound was identified as 1,2,4,5,6,7,8-heptamethyl-3-methylenebicyclo[3.2.1]oct-6-ene (**figure 1.1 A**) which was confirmed by synthesis. The substance was named *sodorifen* following the name of its producing species *Serratia odorifera* 4Rx13 (later renamed to *S. plymuthica*, Weise *et al.* 2014) (von Reuß *et al.*, 2010).

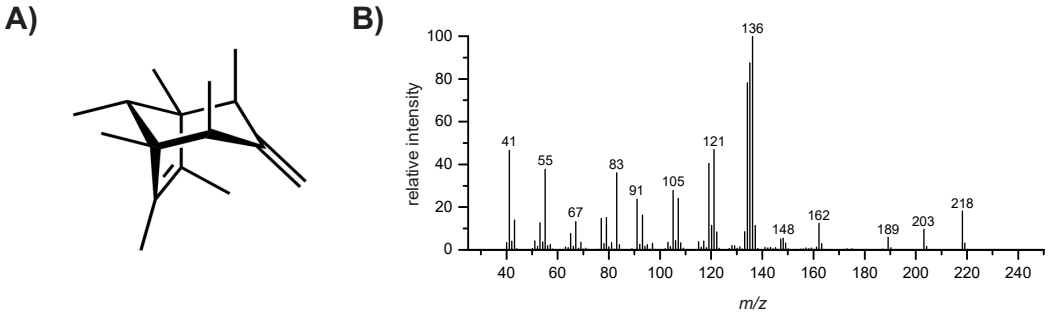

Figure 1.1: Structure and mass spectrum of the unique volatile sodorifen. A) Chemical structure of sodorifen. **B)** Mass spectrum of sodorifen obtained by GC/MS. Modified after von Reuß *et al.* (2010)

Subsequent investigations showed that sodorifen is also produced by other bacterial isolates, all belonging to the species *S. plymuthica* (HRO-C48, 3Re4-18, S13, V4; Domik *et al.* 2016b; Magnus *et al.* 2017). Additionally, several feeding experiments were performed with *S. ply.* 4Rx13 where different carbon sources were applied to minimal medium in order to assess their effects on the sodorifen emission (Magnus *et al.* 2017, von Reuß *et al.*, in preparation). As a result, sodorifen emission varied significantly in response to the media composition. Especially, the use of succinate as sole carbon source resulted in a strong induction of sodorifen emis-

sion, whereas the addition of glucose reduced the emission values to almost zero (Magnus *et al.*, 2017).

Due to its high abundance in the volatile bouquet of *S. ply.* 4Rx13, investigation of the biological function of sodorifen came into focus. Studies, using artificially synthesized sodorifen as a pure substance, neither revealed any phenotypic effect on the model plant *Arabidopsis thaliana* nor on *Serratia marcescens* (Kai & Lemfack, personal communication). The only effect that could be observed so far, was a high toxicity of sodorifen against colorectal carcinoma cell lines (Harms, 2009). But still, the ecological role of this unusual compound remains elusive.

Also chemical classification of sodorifen was difficult at the beginning, therefore the biosynthesis of the unusual volatile was investigated. Since sodorifen was thought to be synthesized via a novel enzymatic pathway, the first attempt was to compare the genomes of different sodorifen producing strains (*S. ply.* 4Rx13, HRO-C48 and 3Re4-18) with other non-producing *Serratia* spp. to identify genes only present in the producer strains (Weise, 2013; Weise *et al.*, 2014; Domik *et al.*, 2016b). Unfortunately, the two identified candidate genes did not exhibit the desired sodorifen-negative phenotype upon knockout mutagenesis (Domik *et al.*, 2016b).

A second approach to investigate sodorifen biosynthesis was performed by transcriptome analysis of *S. ply.* 4Rx13 as well as its close, sodorifen non-producing relative *S. ply.* AS9. Among others, 20 genes were significantly higher expressed in *S. ply.* 4Rx13 than in *S. ply.* AS9 belonging to different metabolic pathways, e.g. carbohydrate metabolism, stress response and nucleotide metabolism (Domik *et al.*, 2016b). Special attention was caught by a gene encoding a putative terpene cyclase (SOD_c20750) which was strongly expressed in *S. ply.* 4Rx13, whereas in *S. ply.* AS9 no expression was detected. Upon knockout of this candidate gene no sodorifen was emitted anymore by *S. ply.* 4Rx13 indicating its crucial role in sodorifen biosynthesis (Domik *et al.*, 2016b). Moreover, the terpene cyclase gene was found to be located in an operon together with three other genes in the genome of *S. ply.* 4Rx13 encoding a methyltransferase (MT, SOD_c20760), DOXP synthase (DXS, SOD_c20770) and an IPP isomerase (SOD_c20780) (**figure 1.2**). Interestingly, also mutagenesis of the MT and IPP isomerase resulted in sodorifen-negative phenotypes (Domik *et al.*, 2016a). Therefore, involvement of this gene cluster in sodorifen biosynthesis (accordingly annotated as sodorifen cluster) became evident and furthermore substantiated the indication that sodorifen belongs to the chemical class of terpenes (Domik *et al.*, 2016a).

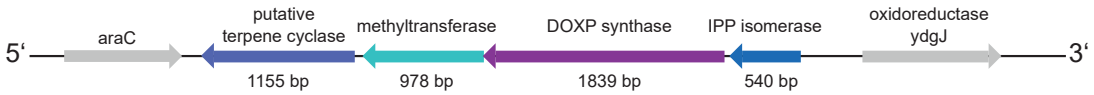

Figure 1.2: Organisation of the sodorifen biosynthesis genes in the genome of *S. ply.* 4Rx13. Genes responsible for sodorifen biosynthesis indicated in colour. Flanking genes of the sodorifen cluster are indicated in grey. Modified after Domik *et al.* (2016a).

1.2.3 Regulation of secondary metabolite biosynthesis

Genes encoding enzymes necessary for secondary metabolite production are often clustered together in operons and are located rather on chromosomal than on plasmid DNA (Demain, 1998; Ruiz *et al.*, 2010). It is known that secondary metabolism is activated during late growth stages of the producing organism (Ruiz *et al.*, 2010). However, certain environmental conditions can lead to premature induction of secondary metabolite production, i.e. nutrient depletion, inducer synthesis/addition, and decreased growth rates (Demain, 1998; Bibb, 2005). Since secondary metabolites derive from the primary metabolism, their syntheses are regulated by similar mechanisms including induction, feedback inhibition and enzyme inactivation (Demain, 1973; Malik, 1979, 1980; Ruiz *et al.*, 2010). Furthermore, nutrient availability plays a crucial role in formation of secondary metabolites. This concerns especially nitrogen, phosphorus and carbon but also metals (summarized in Sanchez *et al.* 2010).

1.2.3.1 Quorum sensing

Biosynthesis of secondary metabolites is often regulated by induction of gene expression. In this case, inducer molecules positively affect gene expression either indirectly by relieving repression of an operon/gene (Barker and Shirley, 1980; Kaempfer and Magasanik, 1967) or directly by activating expression of silent or weakly expressed genes. Direct activation can be executed by small, diffusible molecules, also referred to as autoinducers. They are produced by the receiving organisms themselves and secreted in the surrounding medium. After accumulation to a defined threshold concentration, the autoinducer activates a signaling cascade which ultimately leads to induction of gene expression. This mechanism is called ’quorum sensing’ (QS) and is widely distributed among bacterial species.

In Gram-negative bacteria, N-acyl-homoserine lactones (AHLs) serve as signaling molecules. They are synthesized by an AHL-synthase (encoded by the *lux*I gene and its homologues) and perceived by the corresponding receptor protein (synthesized from the *lux*R gene or homologues). Mostly, the activated QS receptor itself binds to operator sequences, so called ’lux-boxes’, upstream of target genes and

subsequently induces gene expression (Fuqua *et al.*, 1996). Such AHL-dependent QS systems regulate diverse processes, e.g. bioluminescence in *Vibrio fischeri* (**figure 1.3**, Hastings and Greenberg, 1999), animal and plant pathogenicity (Jones *et al.*, 1993; Pearson *et al.*, 1994) and conjugation of the Ti plasmid in *Agrobacterium tumefaciens* (Hwang *et al.*, 1994).

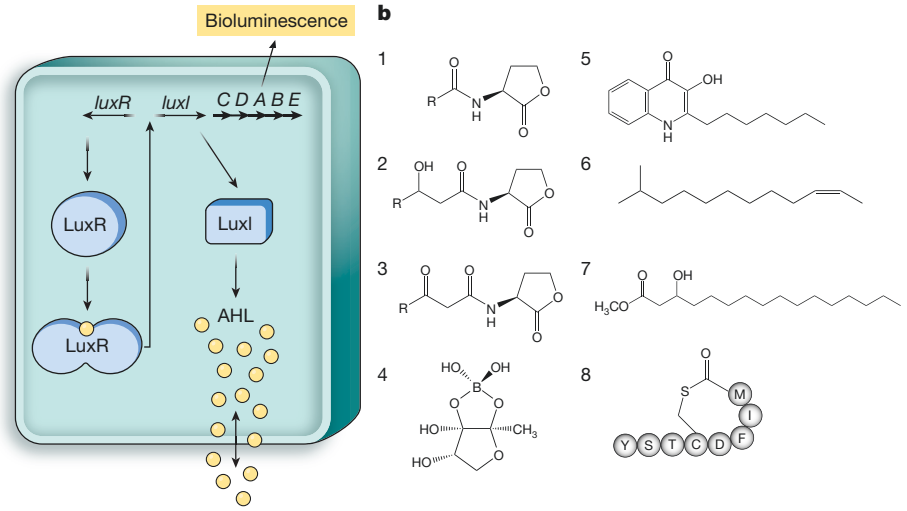

Figure 1 | Canonical QS

Figure 1.3: Mechanism of quorum sensing in *Vibrio fischeri*. LuxI produces 3OC6-HSL (AHL, yellow spheres), which specifically interacts with the LuxR transcriptional regulator when it reaches the threshold concentration. This leads to expression of the *lux*ICDABE operon and finally to bioluminescence. Modified from Whiteley *et al.* (2017).

In the past years, increasing evidence was gained for QS-dependent regulation of bacterial secondary metabolite synthesis. In *Erwinia carotovora* a modified HSL (N-(*β*-ketocaproyl)-L-homoserine lactone, KHL) was shown to be the inducer for biosynthesis of the carbapenem antibiotic. Moreover, the SpnI/R QS system present in *Serratia marcescens* SS-1 leads to cell density-dependent induction of prodigiosin production, a bacterial pigment with a broad range of antagonistic activities (Horng *et al.*, 2002). Other examples of QS-driven gene expression of secondary metabolites have been identified in *Pseudomonas aeruginosa*, and *Chromobacterium violaceum* (Latifi *et al.*, 1995; Ochsner and Reiser, 1995; Winson *et al.*, 1995; McClean *et al.*, 1997).

1.2.3.2 Carbon catabolite repression

In many cases, bacteria use glucose as their favourite carbon source, promoting cell growth and division (Ruiz *et al.*, 2010). However, following the dictum of Demain (1989) "too much of a good thing can be bad", glucose consumption is performed at the expense of little or no secondary metabolite production (Ruiz *et al.*, 2010).

This was found to be due to a mechanism referred to as carbon catabolite repression (CCR). This regulatory system enables bacteria to sequentially take up distinct carbon sources from a mixture according to the preference of the metabolising organism (Sanchez and Demain, 2002). But, as already mentioned, at the same time expression of secondary metabolite synthesizing genes is repressed by the preferred carbon source.

The mechanisms by which carbon catabolite repression takes place are well investigated and differ in Gram-positive and -negative bacteria (summarized in Görke and Stülke 2008). One of the most important pathways by which CCR is executed in Gram-negatives involves the phosphotransferase system (PTS), e.g. of glucose, by which the respective carbon source is transported across the cell membrane and simultaneously phosphorylated to glucose-6-phosphate. The central part of the PTS system consists of three proteins: two are situated in the cell membrane (EIIB/C), contributing the glucose-binding and phosphate-transfer site, and one cytosolic protein (EIIA) is responsible for phosphate transfer to EIIB. The corresponding phosphate group, necessary for glucose transport, is initially provided by dephosphorylation of phosphoenolpyruvate and is subsequently transported to the EIIA-protein via a phosphoryl transfer chain involving the proteins EI and HPr (**figure 1.4**, Sanchez *et al.* 2010). Concerning the mechanism of CCR, EIIA is the central regulator. When glucose concentration is high in the surrounding medium it is taken up and subsequently phosphorylated by EIIB/C. Hence, EIIA, which delivers the phosphate group necessary for glucose internalization, is predominantly found in its dephosphorylated form. Dephosphorylated EIIA itself causes repression of transport systems for other carbon sources, a mechanism also referred to as *inducer exclusion*. When glucose starts to be consumed from the environment, its concentration decreases and causes intracellular accumulation of phosphorylated EIIA (EIIA~P). EIIA~P, in turn, positively affects activity of the adenylate cyclase, an enzyme synthesizing the second messenger cAMP from ATP (Lévy *et al.*, 1990). Subsequently, cAMP forms a complex with its respective receptor (CRP, *cAMP receptor protein*) which is finally capable of binding to promoter regions of its target genes and induce their transcription (Botsford and Harman, 1992; Deutscher, 2008).

Since the discovery of this global regulatory mechanisms, a growing number of CCR-sensitive genes has been identified in microorganisms. Among them, also several operons encoding biosynthetic genes of secondary metabolites were determined. One prominent example is the synthesis of the antibiotic carbapenem in *Erwinia carotovora* and *Serratia* sp. ATCC 39006. In this case, glycerol suppresses carbapenem production indirectly by negative regulation of the quorum sensing system necessary for induction of carbapenem biosynthetic gene expression (Ruiz

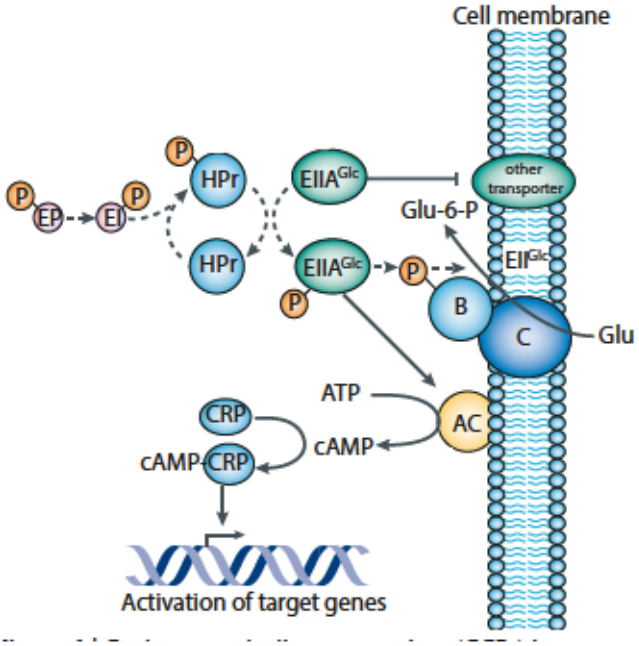

Figure 1.4: Mechanism of carbon catabolite repression in *Escherichia coli*. For detailed description, see text (1.2.3.2). AC = Adenylate cyclase, B/C = part of the PTS, EIIB/C proteins, Glu = glucose, Glu-6-P = glucose-6-phosphate. Modified from Görke and Stülke (2008).

et al., 2010). Furthermore, production of pyocyanin in *Pseudomonas aeruginosa*, actinomycin and cephalosporin in *Streptomyces* spp. was shown to be positively regulated by CCR (Gallo and Katz, 1972; Aharonowitz and Demain, 1978; Huang *et al.*, 2012). In contrast to this, there is also evidence for negative regulation of secondary metabolite synthesis, like in *Serratia marcescens* where prodigiosin production was evidently repressed by elevated cAMP levels (Kalivoda *et al.*, 2010).

In conclusion, CCR can be considered as a global regulatory process capable to affect secondary metabolite production both, positively and negatively.

1.3 Aim of this study

As already mentioned above, sodorifen was found to elicit toxic effects on colorectal carcinoma cells *in vitro*. Therefore, a medical application of this unusual compound could become of great interest in the future, making a large scale production necessary. Furthermore, all studies performed so far could not help to elucidate the ecological function of sodorifen for the producing organism.

In both cases investigation of regulatory mechanisms underlying the biosynthesis of sodorifen can help solving the aforementioned problems. By investigation of how sodorifen emission can be elevated, larger amounts of the volatile can be produced, concentrated and applied in further studies. More importantly, identification

of mechanisms affecting sodorifen biosynthesis can lead to a better understanding of conditions which cause sodorifen emission (e.g. biotic/abiotic stress) and hence allow drawing conclusions to its function in nature.

Therefore, it was the aim of this study to investigate the regulation of sodorifen emission and biosynthesis. Special focus was put on elucidation of i) the level of regulation (i.e. transcription, translation, enzyme activity) and ii) factors positively affecting sodorifen emission. In order to do this, expression analyses of the sodorifen cluster, under different cultivation conditions of the bacteria, were performed by RT-PCR as well as Northern blot. Moreover, the effect of specific regulatory mechanisms (e.g. carbon catabolite repression and quorum sensing) was assessed by insertional deletion mutagenesis of central genes taking part in the respective pathways.

2 Materials and methods

2.1 Organisms and plasmids

2.1.1 Organisms

All wild type bacteria used in this work are listed in **table 2.1**. During this work only bacteria belonging to the species *Serratia plymuthica* (*S. ply.*) were used. The strains 4Rx13 and HRO-C48 were originally isolated from the rhizosphere of oilseed rape (*Brassica napus*), whereas 3Re4-18 was isolated from the endorhiza of potato (*Solanum tuberosum*). The isolate V4 was kindly provided by the research group of R. Kolter (Harvard Medical School, Boston, USA) and *S. ply.* S13 was obtained from Gabriele Berg (Graz University of Technology, Austria).

Table 2.1: Wild type organisms. SCAM Rostock (*Strain Collection of Antagonistic Microorganisms*) Department of Microbiology, University of Rostock, DE. SCAM Graz, Institute of Environmental Biotechnology, Graz University of Technology, Austria.

Genus	Species	Strain	Source	Reference
Serratia	*plymuthica*	4Rx13	SCAM Rostock	Berg *et al.* (2002)
		HRO-C48	SCAM Rostock, DE	Kalbe *et al.* (1996)
		3Re4-18	SCAM Rostock, DE	Berg *et al.* (2005)
		V4	Harvard Medical School, Boston, USA	Cleto *et al.* (2012)
		S13	SCAM Graz	Fürnkranz *et al.* (2012)
		AS9	Swedish University of Agricultural Sciences, Uppsala, Sweden	Alström (2001)

Originating from *S. ply.* 4Rx13 wild type several insertion mutants were constructed using homologous recombination (Muyrers *et al.*, 2000) which are shown in **table 2.3**.

2.1.2 Plasmids

All applied plasmids are listed in **table 2.5**. The vector pJET1.2 was used for sequencing of target DNA, whereas the plasmids pRed/ET and pFRT were part of the commercially available 'Quick and Easy *E. coli* Gene Deletion Kit' (Gene Bridges, 2014) and used for the construction of insertion mutants.

Table 2.3: Insertion mutants. Mutants constructed during this work and their genotypes are listed below. They are available at the laboratory collection of the Department of Biochemistry, University of Rostock, DE.

Organism	Mutant name	Genotype
Serratia plymuthica 4Rx13	*rpo*S::Km	*rpo*S::FRT-PGK-gb2-neo-FRT, Km^R
	cya::Km	*cya*::FRT-PGK-gb2-neo-FRT, Km^R
	crp::Km	*crp*::FRT-PGK-gb2-neo-FRT, Km^R
	CRE1::Km	CRE1::FRT-PGK-gb2-neo-FRT, Km^R
	CRE2::Km	CRE2::FRT-PGK-gb2-neo-FRT, Km^R
	*esa*I::Km	*esa*I::FRT-PGK-gb2-neo-FRT, Km^R
	*esa*R::Km	*esa*R::FRT-PGK-gb2-neo-FRT, Km^R

Table 2.5: Plasmids and vectors. If no host bacterium is mentioned, the plasmid/vector was obtained without previous transformation.

Name	Host	Size (kb)	Properties	Origin
pRed/ET	*E. coli* HS 996	9.27	Tet^R, Redα, Redβ, Redγ, RecA	Gene Bridges, Heidelberg, DE
pFRT	*E. coli* HC 100	3.4	Km^R, FRT-PGK-gb2-neo-FRT cassette	Gene Bridges, Heidelberg, DE
pJET1.2		2.974	Amp^R, T7 promoter, MCS	Thermo Fisher Scientific, Madison, USA

2.2 Media and Cultivation of bacteria

All experiments involving bacteria were performed under sterile conditions (Heraeus Instruments, HS15, Hanau, DE).

2.2.1 Media

Serratia plymuthica isolates and mutants were cultivated either in complex or minimal medium. LB broth (Luria-Bertani, pH 7.2; Bertani 1951) was used as complex medium, containing 10 g/l tryptone, 5 g/l yeast extract and 10 g/l sodium chloride for liquid medium. Through addition of 15 g/l agar-agar solid LB medium was obtained. If necessary an appropriate antibiotic was added to the medium (concentrations given in **table 2.8**). As minimal medium (MM) modified Davis and Mingioli medium (DMM) supplemented with different carbon sources was utilized (7 g/l K_2HPO_4, 3 g/l KH_2PO_4, 0.5 g/l sodium citrate, 0.1 g/l $MgSO_4$ x 7 H_2O, 1 g/l $(NH_4)_2SO_4$, pH 6.2;

Davis and Mingioli 1950). Carbon sources added to the minimal medium and their concentrations are listed in **table 2.7**. After electrotransformation of *S. ply.* 4Rx13, cells were incubated in SOC medium (20 g/l tryptone, 5 g/l yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose; Hanahan 1983) for cell wall recovery and rapid cell division. Before utilization media were sterilized either by autoclaving (LB; 120 °C, 20 min, Varioklav Typ400, H+P Labortechnik GmbH, Oberschleißheim, DE) or by steril filtering (MM; Millipore filters, pore size 0.2 µm, Sarstedt, Nümbrecht, DE).

Table 2.7: Carbon sources used in minimal media.

Carbon source(s)	Concentration
Succinate	55 mM
Glucose	55 mM
Succinate + glucose	each 55 mM
Succinate + met	50 mM + 20 mM
Ala, met, thr Glu, met, thr Iso, Leu, Val	each 20 mM

2.2.1.1 Media additives

For cultivation of bacteria carrying resistance genes an appropriate concentration of the corresponding antibiotic was added to the medium. In the case of solid medium this was done prior to solidification at a temperature of ca. 55 °C, whereas for liquid medium the antibiotic was added directly before application. In the following table (2.8) utilized antibiotics together with their final concentrations are listed. All antibiotics used were obtained from Carl Roth GmbH+Co. KG (Karlsruhe, DE).

Table 2.8: Applied antibiotics.

Antibiotic	Final concentration
Ampicillin	100 µg/ml
Chloramphenicol	15 µg/ml
Kanamycin	15 µg/ml
Tetracycline	10 µg/ml

2.2.2 Cultivation of bacteria

Bacteria were cultivated either on solid medium (see 2.2.2.1) or in liquid medium (as pre- and maincultures; see 2.2.2.2 and 2.2.2.3). LB was used as solid medium.

2.2.2.1 Storage of bacteria

For long term storage of bacteria cryogenic stocks were prepared from precultures (2.2.2.2). After transfer of 500 µl bacterial culture into a sterile 1,5 ml reaction tube, 250 µl of LB medium and glycerol (100 %, v/v) were added, respectively. Subsequently the stock was stored at -70 °C.

The short term storage of bacteria was performed on solid LB medium or solid LB supplemented with the appropriate antibiotics (see tables 2.3, 2.5). Cells were taken from a cryogenic stock and plated on solid medium. After incubation over night at 30 °C (*S. ply.*) and 37 °C (*E. coli*), repsectively, single colonies were obtained, the plate was wrapped with Parafilm ® (Carl Roth GmbH+Co. KG, Karlsruhe, DE) and further stored at 4 °C for up to four weeks.

2.2.2.2 Precultures

For over night cultivation of bacteria in small volumes, precultures were inoculated. Therefore, 6 ml of liquid medium were transferred into a 15 ml tube (Sarstedt, Nümbrecht, DE) and inoculated with a single colony of the respective bacterium using a sterile inoculation loop. Afterwards, the tubes were incubated on a laboratory shaker at 170 rpm and 30 °C for *S. ply.* strains or 37 °C for *E. coli* strains for 12-16 hours. If necessary, antibiotics were added.

2.2.2.3 Main cultures

Main cultures were prepared by inoculating 100 ml of liquid medium with bacteria previously grown in a preculture. The amount of bacteria to be transferred was calculated based upon the optical density at 600 nm (OD_{600}, see 2.2.3) of the preculture to reach a final OD_{600} of 0.005 in the main culture. After inoculation, cultures were incubated for up to 72 h at 170 rpm and 30 °C or 37 °C, depending on the bacterial strain to be cultured.

2.2.2.4 Collection of cell-free supernatants

Originating from main cultures, cell-free supernatants could be obtained. For this purpose, the main cultures were harvested after 24 h of cultivation by centrifugation (10.000 x g, 4 °C, 20 min; SIGMA Laborzentrifugen 3-30KS, Osterode am Harz, DE). The supernatant was transferred into a new tube, steril filtered (Millipore filters, pore size 0.2 µm, Sarstedt, Nümbrecht, DE) and subsequently frozen in liquid nitrogen. Until utilization, the supernatants were stored at -70 °C.

2.2.3 Determination of physiological parameters

The growth of bacterial cultures was monitored using indirect (OD_{600}) and direct (CFU/ml) approaches.

2.2.3.1 Optical density (OD_{600})

The optical density can be used to measure the growth of bacteria indirectly, since the turbidity of bacterial cultures reflects the number of cells present in the medium. Therefore, the optical density at 600 nm of a 1:10 dilution of the bacterial culture was measured against a medium reference (Ultrospec 2000, Pharmacia Biotech, Dübendorf, DE).

2.2.3.2 Living cell number (*colony forming units*, CFU/ml)

In order to directly assess the amount of viable cells, the living cell number was determined as *colony forming units* per ml culture volume (CFU/ml). For this, serial dilutions of the bacterial culture were performed in 0.85 % NaCl and plated on solid LB medium. After incubation over night at 30 °C the number of single colonies was determined in order to calculate the CFU/ml.

2.2.3.3 Analysis of growth behaviour

Furthermore, growth analyses were conducted to exclude variations (e.g. in volatile emission) due to a different growth behaviour of bacteria of distinct species and genotype, respectively. For this purpose main cultures of the relevant bacteria were inoculated and the growth was monitored by measurement of the optical density (30 min interval) and the living cell number (2 h interval) for at least 24 h. Based on the obtained data, growth parameters were calculated and compared. **Equations 2.1, 2.2, 2.3** and **2.4** represent the calculation of growth rate (μ), doubling time (t_d), division rate (v) and generation time (g), respectively. For the calculation only the exponential growth phase was considered.

$$\mu = \frac{log\ x_2 - log\ x_1}{log\ e \cdot (t_2 - t_1)} \qquad x_{1/2} = \text{values at} \qquad (2.1)$$

$$t_{1/2} = \text{timepoints 1 and 2}$$

$$t_d = \frac{ln\ 2}{\mu} \qquad (2.2)$$

$$v = \frac{log\ N_2 - log\ N_1}{log\ 2 \cdot (t_2 - t_1)} \qquad N_{1/2} = \text{cell numbers at} \qquad (2.3)$$

$$t_{1/2} = \text{timepoints 1 and 2}$$

$$g = \frac{1}{v} \qquad (2.4)$$

2.2.3.4 Determination of glucose concentration

The consumption of glucose from the medium during bacterial cultivation was measured using an optical enzyme assay based on Warburg. Thereby, the additional absorption maximum of NADPH+H$^+$ at 340 nm is used to detect the amount of glucose being present in the sample. As shown in **figure 2.1**, glucose is phosphorylated by hexokinase to glucose-6-phosphate at the expense of ATP consumption. Further, glucose-6-phosphate is then transformed into 6-phosphogluconolactone by simultaneous reduction of NADP$^+$ to NADPH+H$^+$. This means that the increase of absorption at 340 nm correlates to the amount of NADPH+H$^+$ being produced and therefore also to the amount of glucose present in the sample.

Robert Fischer 20.02.2012

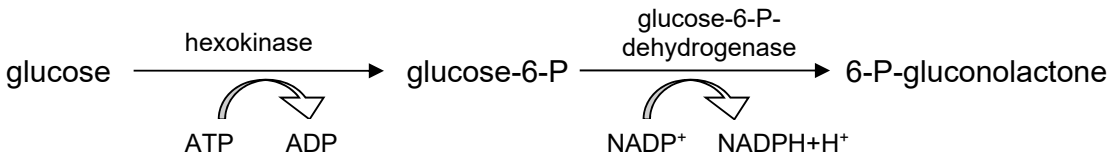

Figure 2.1: Reaction during optical enzyme assay. Während der enzymatischen Messung ermöglicht die Unterscheidung von NADP$^+$ und NADPH+H$^+$. Demnach ist die Menge der gebildeten NADPH+H$^+$ proportional zur Glukosekonzentration der untersuchten Probe. Um die Glukosemenge zu unterschiedlichen Zeitpunkten (0, 24, 48, 72 h) nach Animpfen der Hauptkultur messen zu können, wird jeweils ein 1 ml Aliquot der Kultur entnommen, bei 13.000 g für 2 min zentrifugiert, der Überstand in ein neues Eppendorf-Gefäß überführt und bei -20 °C weggefrohren. Dies dient der Entfernung der in Kultur befindlichen Bakterien, die bei längerem Verbleib im Aliquot bzw. einem weiteren Verbrauch der Glucose im Medium, eventuell zu einer Verfälschung der Glukosewerte führen können. Die Bestimmung der Glukose erfolgt nach folgendem Ansatz:

To perform the assay, 2 ml samples of bacterial main culture were taken at distinct time points after inoculation by centrifugation (2 min, 13.000 x g). Thus, cells were separated from the medium and are no longer capable of consuming glucose. Subsequently, the supernatant was transferred into a new tube and stored at -20 °C until measurement. The reaction mixture for the glucose assay is listed in **table 2.9**.

Table 2.9: Reaction composition of glucose assay.

Ingredient	Volume
0.2 M Tris-HCl + 0.002 M $MgSO_4$	900 µl
$NADP^+$/ATP (Roche Diagnostics Deutschland GmbH, Mannheim, DE)	20 µl
sample	10 µl
Mixing & blank measurement at 340 nm (A_1)	
Hexokinase/Glucose-6-P-dehydrogenase enyzme mix (Roche Diagnostics Deutschland GmbH, Mannheim, DE)	10 µl
Mixing, incubate 5 min at room temperature, extinction measurement at 340 nm (A_2)	

Using **equation 2.5**, the concentration of glucose (c) was calculated enabling analysis of glucose consumption over time.

$$c = \frac{\Delta A \cdot V \cdot MW_{glucose}}{d \cdot v \cdot \epsilon} \qquad \Delta A = A_1 - A_2 \tag{2.5}$$

$$V = \text{total volume of reaction mixture}$$

$$MW_{glucose} = 180.16\ g/mol$$

$$d = cuvettethickness = 1\ cm$$

$$v = \text{samplevolume}$$

$$\epsilon = \text{extinction coefficient of NADH at 340 nm} = 6.3\ l \cdot mmol^{-1} \cdot cm^{-1}$$

2.3 Molecular biological techniques

2.3.1 Isolation of total DNA

Total DNA from bacteria was isolated using the NucleoSpin®Tissue Kit (Macherey-Nagel, Düren, DE) according to the manufacturer's instructions for bacterial cells. The concentration and integrity of the isolated DNA was determined photometrically (Evolution 201 Photometer, Thermo Fisher Scientific, Madison, USA) and by gel electrophoresis (see 2.3.4.1). DNA isolates were stored at -20 °C until further utilized.

2.3.2 Isolation of total RNA

The isolation of bacterial RNA was performed according to a modified protocol for hot-phenol extraction after Oelmüller *et al.* (1990). For this purpose, cell pellets were harvested from main cultures (2.2.2.3) after 24-48 h incubation by centrifugation (10 min, 10.000 x g, 4 °C; SIGMA Laborzentrifugen 3-30KS, Osterode am Harz, DE). The pellets were resuspended in 600 µl cold AE buffer (2.72 g/l sodium acetate x $3H_2O$, 0.372 g/l Na_2EDTA x $2H_2O$, pH 5.5) and subsequently transferred to prewarmed SDS-phenol solution (15 µl 25 % SDS in 1.2 ml phenol, 65 °C). After incubation for 10 min at 65 °C the samples were centrifuged for 45 min at 10.000 x g and 4 °C. The upper, aqueous phase was transferred into a new tube followed by the addition of 100 µl 2 M sodium acetate and 600 µl phenol. Upon inverting, a second centrifugation step was performed (30 min, 10.000 x g, 4 °C). Again, the aqueous phase was transferred into a new tube followed by another phenol extraction. The resulting upper phase was diluted in 0.75 vol. 8 M LiCl and after inverting incubated for 30 min at -20 °C. After mixing, the solution was centrifuged for 10 min at 10.000 x g and 4 °C. The supernatant was discarded and the pellet resuspended in 300 µl DEPC-treated, sterile water (DEPC-H_2O) with subsequent addition of 30 µl 3 M sodium acetate (pH 5.2) and 750 µl cold ethanol. Incubation for 30 min at -70 °C was followed by centrifugation for 10 min at 13.000 x g and 4 °C. The RNA pellet was washed in 70 % ethanol, air-dried and finally resuspended in 40 µl DEPC-H_2O. For all RNA samples the integrity was checked using gel electrophoresis (2.3.4.2) and the concentration was determined photometrically. RNA samples were stored at -70 °C until further utilization.

2.3.3 Isolation of plasmid DNA

Plasmid-DNA was isolated using the commercially available NucleoSpin®Plasmid kit (Macherey-Nagel, Düren, DE) according to the manufacturer's protocol. Finally, the plasmid DNA was stored at -20 °C until further utilization. Additionally, a small aliquot was tested for integrity using gel electrophoresis (2.3.4.1).

2.3.4 Gel electrophoresis

To separate DNA or RNA molecules according to their size and to check the integrity of the samples, gel electrophoresis was used.

2.3.4.1 DNA

For separation of DNA molecules 1 % agarose gels (Carl Roth GmbH+Co. KG, Karlsruhe, DE) were prepared in 1x TAE buffer (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA) containing 14 µg/l ethidium bromide. Prior to electrophoresis, 0.2 vol.of 6x Loading Dye (Thermo Fisher Scientific, Madison, USA) was added to the DNA samples. Additionally, a DNA size standard (GeneRuler™ 1 kb, Thermo Fisher Scientific, Madison, USA) was carried along with the DNA samples to be checked. Unless stated otherwise, DNA gel electrophoresis was performed for 45-60 min at 90 V in electrophoresis chambers (biostep GmbH, Burkhardtsdorf, DE). Documentation was performed using the BIO View Transilluminator UST-20M-8K (biostep GmbH, Burkhardtsdorf, DE).

2.3.4.2 RNA

RNA gel electrophoresis was performed in 1 % agarose gels supplemented with 1x RB buffer (20 mM MOPS, 5 mM sodium acetate, 0.5 mM EDTA) and 3.4 % formaldehyde. RNA samples were prepared for electrophoresis by addition of 2 vol. RNA Loading Dye [6 % 10x RB buffer, 21 % formaldehyde, 60 % formamide, 12 % DNA Loading Dye (Thermo Fisher Scientific, Madison, USA), 1 % ethidium bromide (5 mg/ml stock solution, Carl Roth GmbH+Co. KG, Karlsruhe, DE)] followed by 15 min incubation at 65 °C to resolve secondary structures. RNA gel electrophoresis was performed in electrophoresis chambers (Bio Rad, California, USA) for 90 min and 68 V.

2.3.5 Polymerase chain reaction (PCR)

For amplification of specific DNA fragments, polymerase chain reaction (PCR) was used. Therefore, two different types of DNA polymerases were used, Taq- and Phusion-polymerase (Thermo Fisher Scientific, Madison, USA), respectively. The reaction compositions are listed in **tables 2.11** and **2.13**. A total volume of 25 µl for Taq-based PCR and 50 µl for Phusion-based PCR was used. The reactions were conducted in thermal cyclers from Bio Rad (Californie, USA) and Biometra (Göttingen, DE) according to the protocols given in **tables 2.15** and **2.17**. For direct screening of bacteria, colony PCR was conducted by heating bacterial cells (e.g. colony) in 50 µl of ddH_2O for 10 min at 95 °C followed by centrifugation at 10.000 x g for 2 min and 4 °C. Afterwards, 20 µl of the supernatant were discarded and the pellet was resuspended in the residual water. This suspension was used as the DNA template in a Taq-based PCR (see table 2.11). For amplification, primers specific for

the DNA of interest were necessary. All primers were ordered from Sigma-Aldrich (Munich, DE) and a complete list of all primers used in this work can be found in the supplementary part (**supplementary table S1**).

Table 2.11: Reaction composition of Taq-based PCR.

Ingredient	Volume (in µl)
10x Taq-buffer (750 mM Tris-HCl, 200 mM $(NH_4)_2SO_4$, 0.1 % Tween 20, 20 mM $MgCl_2$)	2.5
dNTPs (10 mM each)	1
sense Primer (10 pmol/µl)	1
antisense Primer (10 pmol/µl)	1
DNA template	1
Taq-polymerase	0.5
ddH_2O	18
total volume	25

Table 2.13: Reaction composition of Phusion-based PCR.

Ingredient	Volume (in µl)
5x HF-buffer	10
dNTPs (10 mM each)	1
sense Primer (10 pmol/µl)	1
antisense Primer (10 pmol/µl)	1
DNA template	1
Phusion-polymerase	0.5
ddH_2O	35.5
total volume	50

2.3.6 Reverse transcription polymerase chain reaction (RT-PCR)

To be able to determine the expression of genes, reverse transcription PCR was used. Thereby, conversion of RNA molecules into corresponding cDNA is achieved, which can further be subjected to a PCR reaction. For this purpose, isolated RNA (see 2.3.2) was first treated with DNaseI (Invitrogen, California, USA) according to the manufacturer's instructions. Secondly, the RNA samples were converted into their corresponding cDNA following the protocol given in **table 2.19**. Additionally, a negative control was used by replacing the reverse transcriptase with DEPC-H_2O to

Table 2.15: PCR program for Taq-based reactions. The applied annealing temperature (x) depends on the primers used. For more information see supplementary table S1

.

Reaction step	Time	Temperature	
Denaturing	3 min	95 °C	
Denaturing	1 min	95 °C	30 cycles
Primer annealing	30 sec	x °C	
Elongation	1 min/kb	72 °C	
Final elongation	10 min	72 °C	
Hold	∞	10 °C	

Table 2.17: PCR program for Phusion-based reactions. The applied annealing temperature (x) depends on the primers used. For more information see table S1.

Reaction step	Time	Temperature	
Denaturing	1 min	98 °C	
Denaturing	30 sec	98 °C	30 cycles
Primer annealing	30 sec	x °C	
Elongation	30 sec/kb	72 °C	
Final elongation	10 min	72 °C	
Hold	∞	10 °C	

check for residual DNA. Finally, the resulting cDNA was amplified using a Taq-based PCR (2.11) with 2 µl of cDNA as template. The final result was visualized using DNA gel electrophoresis (2.3.4.1).

Table 2.19: Protocol for RT-PCR.

Component	Volume
RNA (DNaseI-digested)	10 µl
antisense Primer (10 pmol/µl)	2 µl
dNTPs (10 mM each)	1 µl
DEPC-H_2O	1.5 µl
5 min, 65 °C, 1 min on ice	
5x RT-buffer (Thermo Fisher Scientific, Madison, USA)	4 µl
Ribolock (Thermo Fisher Scientific, Madison, USA)	0.5 µl
Maxima Reverse Transcriptase (Thermo Fisher Scientific, Madison, USA) or DEPC-H_2O (negative control)	0.4 µl
DEPC-H_2O	0.6 µl
30 min, 50 °C 5 min, 85 °C 1 min on ice	

2.3.7 Rapid amplification of 5' cDNA ends (5' RACE)

The determination of the transcriptional start site of an operon or gene was conducted using the 5'-RACE technique. A protocol developed by Scotto-Lavino *et al.* (2006) was applied. As a first step RT-PCR (2.3.6) was performed after DNaseI digest of total RNA to produce cDNA of the gene of interest. Instead of subjecting this cDNA to a Taq-based PCR, the RNA template was destroyed (using RNaseH) followed by poly-(A)-tailing of the cDNA. Through this step, amplification of 5' cDNA ends was possible using specific primers (GSP1, GSP2, Q_T, Q_O & Q_i) in two successive rounds. A detailed protocol of the procedure can be found in **supplemental table S2**. Afterwards, the result was checked via gel electrophoresis and the final PCR product was sequenced (2.3.11).

2.3.8 DNA Clean-up

Purification of DNA samples, either from agarose gels or after PCR reactions, was conducted with the NucleoSpin®Gel and PCR Clean-up Kit (Macherey-Nagel, Düren, DE) according to the manufacturer's instructions. But, instead of elution with the provided buffer, ddH_2O was used.

2.3.9 Northern blot

For quantitative evaluation of gene expression levels, the Northern blot technique was applied. Therefore, gene-specific, labelled probes were generated which later can be detected using chemiluminescence.

2.3.9.1 Generation of DIG-labelled DNA probes

As a labelling agent digoxigenin-11-UTP (DIG-dUTP, Roche Diagnostics Deutschland GmbH, Mannheim, DE) was incorporated into a DNA fragment complementary to the gene of interest. This is done by PCR using the protocol presented in **table 2.21**. For amplification, the PCR program for Taq-based reactions (2.15) was used with the number of performed cycles increased to 40.

2.3.9.2 Performing a Northern blot

In a first step, 5 µg of total RNA were separated using RNA gel electrophoresis (2.3.4.2). Afterwards, a capillary blot was performed with two layers of filter paper at the bottom, covered by the RNA gel, a nylon membrane (Carl Roth GmbH+Co. KG, Karlsruhe, DE), two layers of Whatman paper (Carl Roth GmbH+Co. KG, Karlsruhe,

DE) and a seven cm layer of paper towels. On top, ca. 100 g of weight were put. To transfer the RNA from the gel onto the membrane, 20x SSC buffer (3 M NaCl, 0.3 M sodium citrate, pH 7.0) was applied in a bowl with ends of filter paper hanging in. Due to capillary forces, the buffer is drawn through the gel and membrane into the paper towels and thereby RNA is transferred onto the membrane. After incubation of the capillary blot over night, the transferred RNA was crosslinked to the membran using UV radiation (Techne CL-508.G, thermo-DUX GmbH, Wertheim, DE). Following this, the membrane was washed for 1 h in prehybridization buffer (5x SSC, 50 % formamide, 7 % SDS, 0.1 % N-lauroylsarcosine, 0.2x Blocking solution, 0.05 M Na_2HPO_4) and 40 ng of the DIG-labelled probe (2.3.9.1) were applied. After incubation over night at 55 °C, the blot was detected. First, it was washed two times in 2x SSC + 0.1 % SDS for 5 min and two times, 15 min in 0.1x SSC + 0.1 % SDS, both at room temperature. Secondly, vacant sites on the membrane were blocked for 1 h at 55 °C (1x Blocking solution). Thirdly, 0.75 U of anti-DIG-antibody (anti-Digoxigenin-AP fab fragments, Roche Diagnostics Deutschland GmbH, Mannheim, DE) were applied for 30 min at room temperature to detect the hybridized DIG-probes. Subsequently, the blot was washed two times for 15 min in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.0) supplemented with 0.3 % Tween20 (Carl Roth GmbH+Co. KG, Karlsruhe, DE) and 2 min in Detection buffer (0.1 M Tris base, 0.1 M NaCl, 50 mM $MgCl_2$ x 6 H_2O, pH 9.5). Finally, CDP-Star (37.2 µg, Roche Diagnostics Deutschland GmbH, Mannheim, DE) was applied and incubated for 5 min at room temperature. After removing residual solution, the result was documented by chemiluminescence (STELLA 3200, raytest, Straubenhardt, DE).

2.3.10 Insertional deletion mutagenesis

Site-specific inactivation of *S. ply.* 4Rx13 genes was achieved using the Quick & Easy *E. coli* Gene Deletion Kit (Gene Bridges, Heidelberg, DE). Thereby, a func-

Table 2.21: Construction of DIG-labelled probes.

Component	Volume
10x Taq buffer	5 µl
sense Primer	2 µl
antisense Primer	2 µl
dATP, dCTP, dGTP (10 mM)	each 1 µl
dTTP (1 mM)	6.5 µl
DIG-dUTP	1 µl
Taq polymerase	1 µl
DNA template	1 µl

tional cassette, containing a kanamycin resistance gene, (FRT-PGK-gb2-neo-FRT; supplied by helper plasmid pFRT) is inserted into the *open reading frame* (ORF) of a gene by homologous recombination, causing its disruption. The process of recombination is supported by a helper plasmid (pRed/ET) provided by the kit. This plasmid contains genes encoding for Redα, β and γ under the control of an arabinose-inducible promoter. In a previous study (Weise, 2013) pRed/ET was already transformed into *S. ply.* 4Rx13.

2.3.10.1 Preparation of electrocompetent cells

Prior to homologous recombination of the functional cassette into the gene of interest, the generated PCR construct has to be transformed into the organism of interest (in this case *S. ply.* 4Rx13). For this purpose, electroporation was used, making the preparation of electrocompetent *S. ply.* 4Rx13 cells indispensable. A modified protocol from Untergasser was used, and already established for *S. ply.* 4Rx13 in a previous work (Weise, 2013).

2.3.10.2 Construction and transformation of functional cassette

In order to achieve recombination, homologous regions to the gene of interest have to be attached to the functional cassette. This is performed by PCR where the designed primers contain regions specific for the amplification of the functional cassette (23 bp) and homologous to the gene of interest (50 bp). To generate these gene-specific functional cassettes, a Phusion-based PCR (tables 2.13 & 2.17) was performed using pFRT as a template and 73 bp long primers containing 50 bp of homologous regions of the gene to be knocked out (see table S1). The resulting PCR product was cleaned up and used for subsequent electroporation into competent cells of *S. ply.* 4Rx13 + pRed/ET. Therefore, one 40 µl aliquot of competent cells was thawed on ice and incubated together with 1 µl of the generated functional cassette for 30 min. After transfer to electroporation cuvettes (0.2 cm electrode distance, Bio Rad, California, USA) an electric pulse (2.5 kV, 200 Ω, 25 µF) was applied, followed by addition of 800 µl SOC medium and further incubation for 4 h at 37 °C and 170 rpm. The cells were pelleted (2 min, 11.000 x g, FRESCO 17, Thermo Fisher Scientific, Madison, USA) and entirely plated on solid LB medium containing kanamycin to select for successful integration of the functional cassette. After incubation at 37 °C over night, positive clones were picked and checked for the correct insertion using colony PCR (2.3.5).

2.3.11 DNA sequencing

DNA sequencing was performed by GATC Biotech AG (Konstanz, DE) using Sanger sequencing technique. Primers were either provided by GATC or Sigma-Aldrich (see table S1).

2.4 Analysis of volatile organic compounds (VOCs)

Analysis of volatiles emitted by bacterial cultures was performed by collecting the VOCs on an adsorbent, followed by subsequent elution and measuring by coupled gas chromatography and mass spectrometry.

2.4.1 Collection of VOCs

Bacterial volatiles were collected using the closed VOC collection system (modified after Kai *et al.* 2010, see **figure 2.2**). Therefore, 250 ml Erlenmeyer flasks were provided with ground-glass stoppers to hermetically seal the flasks, and two opposing outlets. At one side of the flask, charcoal and cotton filters are connected to ensure entering of VOC-free, sterile air. On the other side, a glass column filled with 20 µg of the adsorbent material (Porapak™, Sigma-Aldrich, Munich, DE) is placed between two layers of cotton (50 µg each) which, in turn, is connected to a membrane pump (Denver Gardner, Puchheim, DE). As connectors, inert Isoversinic® tubes were applied (Carl Roth GmbH+Co. KG, Karlsruhe, DE). When power is applied to the pump, air is sucked through the filters and enriched with volatiles emitted from the bacterial culture growing inside the flask. Subsequently, the VOCs are collected on the adsorbing material and can be eluted from the column. Elution is performed by rinsing the column two times with 200 µl and 100 µl dichloromethane (CH_2Cl_2, Carl Roth GmbH+Co. KG, Karlsruhe, DE), respectively. Prior to elution, nonylacetate (5 ng/µl, ABCR GmbH & Co. KG, Karlsruhe, DE) is applied onto the column as internal standard. The resulting eluate was used for measurement of the collected volatiles by gas chromatography and mass spectrometry. For investigating the volatile spectrum of a bacterial strain, cultures were inoculated in the modified 250 ml Erlenmeyer flasks according to 2.2.2.3 and incubated for up to 96 h on a magnetic stirrer at 250 rpm. Volatiles were eluted every 24 h from the column and the living cell number was determined simultaneously.

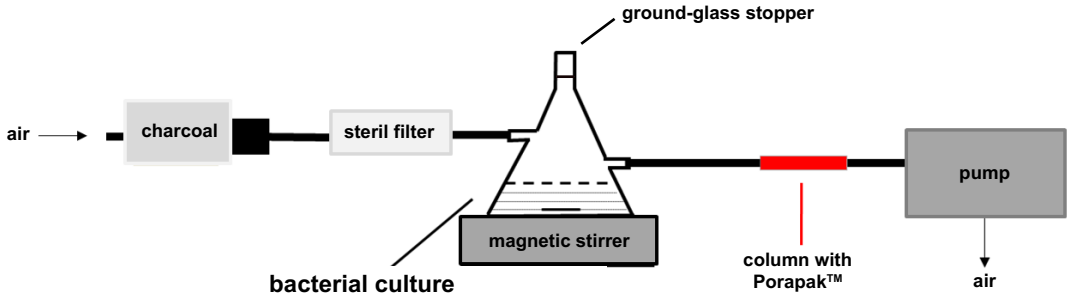

Figure 2.2: Assembly of the closed VOC collection system. Modified after Kai *et al.* (2010).

2.4.2 Analysis of collected VOCs by gas chromatography mass spectrometry

Collected volatiles were analyzed using the gas chromatograph - mass spectrometer (GC-MS) QP 5000 (Shimadzu Deutschland GmbH, Duisburg, DE). Therefore, 1 µl of the eluted volatile-solvent mixture was injected by the autosampler into the gas chromatograph. Following this, the sample was vaporized and transferred, either splitless or with a split ratio of 1:10, into a 60 m long capillary column, coated with (diphenyl-)(dimethyl-)polysiloxane and (5 % phenyl-)methylpolysiloxane (DB-5, Agilent Technologies, California, USA) as stationary phase using helium as carrier gas. The separated compounds were exposed to Electron Impact (EI) ionization in the mass spectrometer and fragmented according to their mass-charge ratio. The selection of the fragments was conducted using a quadrupole mass analyzer. Finally, the detected masses were analyzed using the LabSolutions Software (v. 1.20) from Shimadzu Deutschland (Duisburg, DE) and identified using the NIST107 mass spectral library (National Institute of Standards and Technology, Maryland, USA). The parameters used for GC-MS analysis of VOC eluates are listed in **table 2.22**.

Table 2.22: Parameters for GC-MS analyses.

Function	Value
Sampling time	2 min
Injection temperature	200 °C
Interface temperature	300 °C
Control mode	splitless
Column flow	1.1 ml/min
Linear velocity	27.2 cm/sec
Split Ratio	10
Total flow	16.1 ml/min
Temperature program	35 °C, 2 min hold, 10 °C/min - 280 °C, 15 min hold
Program time	41.5 min
Aquisition mode	Scan 41-280 m/z
Detector voltage	1.3 kV
Treshhold	1000
Interval	0.5 sec
Solvent cut time	7.5 min

3 Results

The rhizobacterium *Serratia plymuthica* 4Rx13 is one of few bacterial strains capable of emitting the new and unusual volatile compound sodorifen. According to latest results, sodorifen belongs to the chemical class of terpenes (Domik *et al.*, 2016a) but still nothing is known about the biological function of this volatile. One strategy to unravel its function is the analysis of regulatory processes underlying the feature of sodorifen emission. In this work, first results concerning the regulation of sodorifen emission are presented, revealing a mulitlayered network consisting of transcriptional and post-transcriptional regulatory processes.

3.1 Sodorifen emission in *Serratia* spp.

Hitherto, five bacterial strains capable of emitting sodorifen have been identified including *Serratia plymuthica* 4Rx13, HRO-C48, 3Re4-18, S13 and V4. In this section, the amount of sodorifen emitted by different *Serratia* strains was compared and levels of regulation were determined. Subsequently, the generated results provided the basis for further research concerning differences in the regulation of sodorifen emission.

3.1.1 Quantitative comparison of sodorifen emission in different sodorifen-producers

Comparison of the sodorifen amounts emitted by the strains *S. ply.* 4Rx13, HRO-C48, 3Re4-18, S13 and V4 were conducted using the closed VOC collection system followed by GCMS analysis (2.4.1, 2.4.2). The bacteria were cultivated in complex medium (2.2.1) inside the collection system for 72 h with concurrent elution of the volatiles every 24 h. **Figure 3.1** represents the relative sodorifen emissions determined in the different producer strains. The sodorifen emission in *S. ply.* 4Rx13 after 24 h of cultivation was set to 100 % and the values of the other producer strains were calculated relative to this. Thus, it became apparent that at all time intervals tested, *S. ply.* 4Rx13 reached the highest amounts of sodorifen emission. Interestingly, all other producers achieved only 0.1-14.5 % of sodorifen emission relative to 4Rx13. In addition, the relative sodorifen emissions in *S. ply.* HRO-C48, 3Re4-18 were almost equal with values around 5 % at 0-24 h and finally decreasing to a minimum of about 1-2 %. V4 produced the least amounts of sodorifen (0.1-1.8 %), whereas HRO-C48 produced up to 14.5 % sodorifen compared to 4Rx13. The fact that the

sodorifen emission in *S. ply.* 4Rx13 exceeds that of the other producers about 15 to 1300-fold, indicates a diverging regulation of the sodorifen emission in the different strains tested.

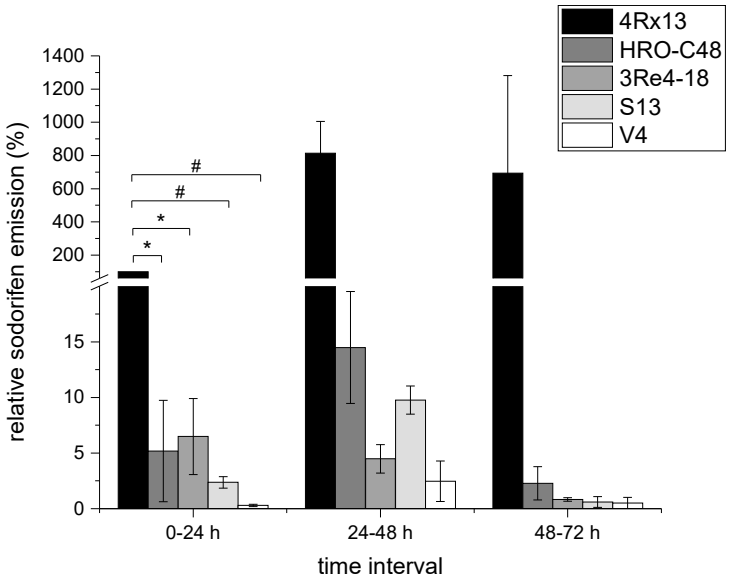

Figure 3.1: Relative sodorifen emission of the producer strains. The sodorifen emission of the producer strains *S. ply.* 4Rx13, HRO-C48, 3Re4-18, S13 and V4 was determined during growth in complex medium. Relative sodorifen emission was calculated in relation to the living cell number, with *S. ply.* 4Rx13 after 24 h of cultivation representing 100 %. Error bars indicate standard deviation (n=3). * $p < 0.05$; # $p < 0.01$.

3.1.2 Expression of the sodorifen cluster genes in *Serratia* sp.

In previous studies, a cluster of four consecutive genes was identified in *S. ply.* 4Rx13 which is evidently involved in the sodorifen biosynthesis (Domik *et al.*, 2016a). Furthermore, comparative transcriptome analysis of the sodorifen producer *S. ply.* 4Rx13 and the non-producer strain AS9 revealed that this gene cluster was only expressed in the sodorifen-producing isolate. These four cluster genes code for an isopentenyl diphosphate (IPP) isomerase, a DOXP synthase (DXS), a methyltransferase and a terpene cyclase encoded by the genes SOD_c20780, SOD_c20770, SOD_c20760 and SOD_c20750, respectively. Due to their similar orientation in the genome of *S. ply.* 4Rx13, it was assumed that all four genes are co-transcribed, leading to one long mRNA. To verify this hypothesis, RT-PCR was conducted with total mRNA, isolated from 4Rx13 after 24 h cultivation in minimal medium supplemented with succinate, as a template. A negative control was performed by adding no reverse transcriptase to the reaction setup to ensure that no DNA contamination was present in the RNA samples. Furthermore, the 16 S rRNA was amplified

as a positive control. To prove the existence of one large mRNA molecule, specific primer combinations were used to amplify the total range of all four sodorifen biosynthesis genes as well as parts of it (**figure 3.2 A**). It was possible to determine transcripts comprising the IPP isomerase alone (lane 2; 258 bp), and also the extending combinations of IPP isomerase + DXS (lane 3; 2222 bp), IPP isomerase to methyltransferase (lane 4; 3258 bp) and IPP isomerase to terpene cyclase (lane 5; 4352 bp). As a result, the obtained amplification products showing the expected size, prove the assumed co-transcription of the sodorifen cluster genes.

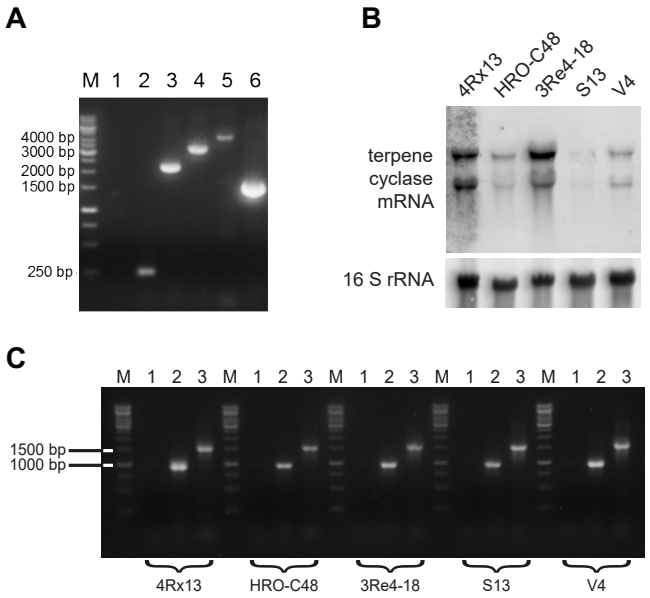

Figure 3.2: Organization and expression of the sodorifen cluster genes. A) Co-transcription of the sodorifen cluster genes. RT-PCR of *S. ply.* 4Rx13 was performed after 24 h cultivation in minimal medium + 55 mM succinate. Lanes 1 and 6 represent the negative (no reverse transcriptase) and positive control (16 S rRNA), respectively. Lanes 2-5 show the transcript of the sodorifen cluster with increasing numbers of genes, beginning with the first gene of the cluster (IPP isomerase, SOD_c20780, lane 2) and ending with the complete sodorifen cluster (SOD_c20750-SOD_c20780, lane 5). Lanes 3 and 4 show the transcripts of the genes SOD_c20770-SOD_c20780 and SOD_c20760-SOD_c20780, respectively. **B)** Expression levels of the terpene cyclase in the sodorifen producer strains *S. ply.* 4Rx13, HRO-C48, 3Re4-18, S13 and V4. Northern blot was performed using total RNA of the respective strains after 24 h cultivation in minimal medium + 55 mM succinate. A DIG-dUTP labelled probe, specific for the terpene cyclase mRNA, was used (upper panel). As a control, a labelled probe detecting the 16 S rRNA was applied afterwards (lower panel). Probe hybridizations were visualized by fluorescence measurements for 1 min. **C)** Expression of the terpene cyclase in *S. ply.* 4Rx13, HRO-C48, 3Re4-18, S13 and V4 after 24 h cultivation in minimal medium supplemented with succinate detected by RT-PCR. Lane 1 represents the negative control, lane 2 shows expression of the terpene cyclase and in lane 3 the positive control (16 S rRNA) is depicted. M: marker GeneRuler 1 kb (Thermo Fisher Scientific, Madison, USA).

As already presented in figure 3.1, there are variations in the amount of sodorifen emitted by the different producer strains. One possible explanation for these dispar-

ities might be the differential expression level of the sodorifen cluster genes. First, RT-PCR was performed on the terpene cyclase gene of the sodorifen cluster in each of the five sodorifen producers known. As can be seen in **figure 3.2 C** it was possible to obtain amplification products of the terpene cyclase (lane 2) with the expected size of 963 bp. Furthermore, the negative controls (lane 1) proved that there was no contaminating DNA present in the reaction mixtures and the equal signal strength of the 16 S rRNA (lane 3) gives evidence to the same amounts of RNA applied. Therefore, it can be concluded that the sodorifen cluster is expressed in all producer strains after 24 h cultivation in minimal medium supplemented with succinate. To be now able to quantify the expression of the sodorifen cluster, Northern blots were performed (2.3.9). Thereby, 5 µg of RNA from each sodorifen producer strain tested were separated in a denaturing RNA agarose gel (2.3.4.2). After transfer of the RNA molecules onto a nylon membrane, a DIG-labelled probe specific for the terpene cyclase gene was applied and its hybridization detected by fluorescence. Afterwards, the same blot was used for a second hybridization with a probe specific for the 16 S rRNA to ensure equal RNA loading on the membrane. Quantification of the signals was performed using the AIDA Image Analyzer software (v.4.50, raytest, Straubenhardt, DE). In case of the probe specific for the terpene cyclase, the fluorescence intensity of the upper band was measured and the intensity from *S. ply.* 4Rx13 was used as a reference and set to 100 %. From **figure 3.2 B** it became obvious that with equal RNA amounts applied (lower panel), there are significant differences in the signal strengths of the terpene cyclase gene (upper panel). Quantification of the expression levels showed that the strongest signals for the terpene cyclase expression were detected in *S.p.* 4Rx13 (100 %) and 3Re4-18 (129 %). Surprisingly, sodorifen cluster expression in *S.p.* 3Re4-18 exceeded the level in 4Rx13 by almost one third despite the significantly lower sodorifen emission in 3Re4-18 (6.5 %; figure 3.1). As expected, the other producers (*S.p.* HRO-C48, *S.p.* S13 and *S.p.* V4) only showed a weak signal for this gene (ca. 2-18 %) in comparison to *S.p.* 4Rx13.

Additionally, the expression level of the sodorifen cluster was assigned during the different growth stages of the bacteria. For this purpose, RNA was isolated from the sodorifen producer strains after 24 h, 48 h and 72 h cultivation in minimal medium supplemented with succinate and again hybridized with a labelled probe specific for the terpene cyclase of the sodorifen cluster. The fluorescence value of *S. ply.* 4Rx13 after 24 h cultivation was set to 100 % and all other values were calculated relative to this. The resulting Northern blot (**figure 3.3**) shows that in *S. ply.* 4Rx13 the signal for the terpene cyclase probe is decreasing over time. After 48 h cultivation the relative fluorescence in 4Rx13 was reduced from 100 % to 5.9 % and was no

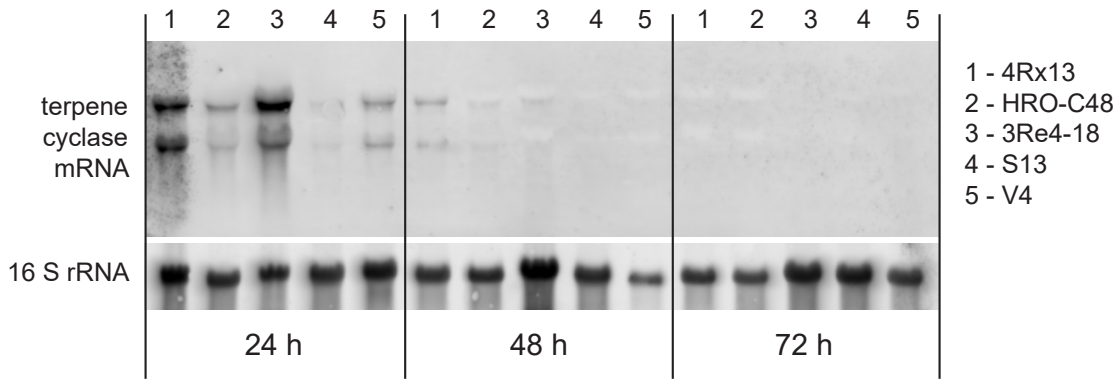

Figure 3.3: Expression of the terpene cyclase in the sodorifen producer strains throughout the different growth stages. Northern blot of *S. ply.* 4Rx13 (lane 1), HRO-C48 (lane 2), 3Re4-18 (lane 3), S13 (lane 4) and V4 (lane 5) after 24 h, 48 h and 72 h cultivation in minimal medium supplemented with 55 mM succinate. A DIG-dUTP labelled probe specific for the terpene cyclase mRNA was hybridized to 5 µg of total RNA for each sample (upper panel). As a control, a labelled probe detecting the 16 S rRNA was applied afterwards (lower panel). Probe detection was performed by fluorescence measurement for 1 min.

longer detectable after 72 h of growth. A decrease in signal strength can also be seen in the case of the other producer strains. In *S. ply.* HRO-C48 signal strength decreased almost 40-fold from 18.5 % after 24 h to only 0.5 % after 48 h cultivation. The most significant decrease was found in 3Re4-18 from 126 % (24 h) to 2.3 % (48 h). Terpene cyclase expression in *S. ply.* S13 and V4 was only detectable after 24 h cultivation reaching intensities of 2.2 % and 12 %, respectively. In conclusion, strongest expression for the terpene cylase genes of all producers were obtained in the first growth interval (0-24 h) which included the exponential as well as beginning of the stationary phase, while thereafter all levels decreased until they were no longer detectable.

Furthermore, previous experiments were concerned with the determination of the exact starting point of sodorifen emission and cluster expression by *S. ply.* 4Rx13 in succinate minimal medium using the VOC collection system as well as RT-PCR (Bernhardt, 2015). As a result, sodorifen emission was first detectable after 13 h incubation. In contrast to this, expression of the sodorifen cluster genes already started after only 5 h of growth, as determined by RT-PCR (Bernhardt, 2015). To be able to quantify the expression levels of the sodorifen biosynthesis genes, Northern blots were performed using RNA isolated at different time points during exponential growth of *S. ply.* 4Rx13 (6 h, 8 h, 9 h, 10 h, 12 h 24 h; **figure 3.4**). At the time points from 6 h to 10 h no terpene cyclase mRNA was detectable. Only after 12.5 h, the transcripts became visible (set to 100 %) with a signal strength equal to the one after 24 h cultivation (106 %). As expected, no terpene cyclase mRNA was detectable in

the sodorifen non-producing strain *S. ply.* AS9 which served as a negative control in this experiment.

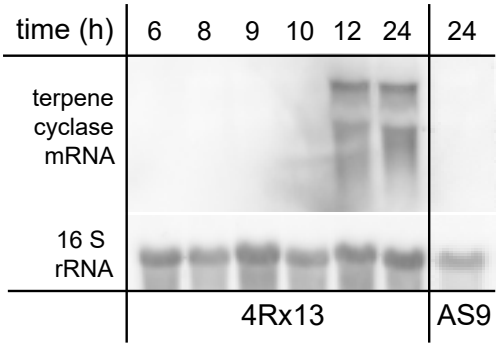

Figure 3.4: Expression of the terpene cyclase in *S. ply.* 4Rx13 during the exponential growth phase. Northern blot was performed with 5 µg RNA isolated from *S. ply.* 4Rx13 after 6 h, 8 h, 9 h, 10 h, 12 h, and 24 h incubation in minimal medium supplemented with 55 mM succinate. Total RNA of the sodorifen non-producer *S. ply.* AS9 was used as a negative control. Expression of the terpene cyclase gene was detected using DIG-dUTP-labelled probes and fluorescence measurement for 1 min (upper panel). As a positive control, the expression of 16 S rRNA was detected in the same manner (lower panel).

3.1.3 Comparison of the sodorifen cluster 5' UTR in different *Serratia* spp.

The results presented in figure 3.2 provide sufficient indications that distinct regulatory processes not only exist between sodorifen producers and non-producers but also among the different producer strains themselves. For this reason, the nucleotide sequence directly upstream of the sodorifen cluster in *S. ply.* 4Rx13 was compared with the respective sequences of the other producers strains *S. ply.* HRO-C48, 3Re4-18, S13 and V4, as well as with the non-producer AS9. The sodorifen cluster upstream sequence was defined as the total number of nucleotides present between the first gene of the sodorifen cluster (in *S. ply.* 4Rx13 = SOD_c20780; IPP isomerase) and the last gene prior to the cluster (in *S. ply.* 4Rx13 = SOD_c20790; putative oxidoreductase *ydgJ*). In 4Rx13, this sequence was 480 bp in length. In the other sodorifen producer strains, the length was almost equal showing 488 bp in HRO-C48 and S13, 479 bp in 3Re4-18 and 480 bp in V4. Contrary to this, the upstream sequence in the sodorifen non-producer *S. ply.* AS9 was only 456 bp in length. Whereas among the sodorifen producers mainly substitutions and small insertions took place in the upstream sequence (see **figure 3.5**), there were two large deletions to be found in AS9 ranging from nucleotides 163-174 and 329-345 (**figure 3.6**) leading to the smaller size. Furthermore, about 17 % of the 4Rx13 sodorifen cluster upstream sequence was altered in AS9 due to nucleotide substitutions. The total identity of the AS9 upstream sequence in relation to 4Rx13 is 74.79 %

and therefore the lowest in comparison to the other sodorifen producer strains with 3Re4-18 showing the highest identity to 4Rx13 with almost 98 %. From figure 3.5 it becomes apparent that the sodorifen producers differ mostly at the same positions from the 4Rx13 sequence (for details see **table S3**). The upstream nucleotides of the strains HRO-C48 and S13 are identical to each other. Also 3Re4-18 is very close to these two strains with the exception that the last nine nucleotides are missing. The upstream sequence most distinct from 4Rx13 among the sodorifen producer strains can be found in *S. ply.* V4 with an identity of 94.38 %. Here, additional to several substitutions, a single guanine was inserted at position 149 leading to a shift in the upstream sequence. Also in S13 and HRO-C48 an insertion of eight nucleotides appeared, however, in contrast to V4, not in the middle of the sequence but at the very end. Taken together it becomes apparent that all sodorifen producer strains share a high homology of the sodorifen cluster upstream sequences with 3Re4-18 having the highest identity, whereas the non-producer isolate AS9 shows least identity to *S. ply.* 4Rx13.

3.1.4 Determination of the transcriptional start site of the sodorifen cluster and promoter analysis

For determination of possible regulatory proteins that can bind upstream of the sodorifen cluster, the transcriptional start site was analyzed. For this purpose, 5'-RACE-PCR was performed. As a first step, total RNA from *S. ply.* 4Rx13 was used as a template to generate cDNA with a reverse primer for the first gene of the sodorifen cluster (IPP isomerase). The resulting amplification product was then polyadenylated and used again as a template in a second round of PCR with a primer containing a poly-(T) tail and a gene-specific primer. Finally, a third set of amplifications was performed with nested primers to increase the yield of the resulting products (Scotto-Lavino *et al.*, 2006). In the end, the obtained fragment was sequenced and compared to the genomic sequence of *S. ply.* 4Rx13 to determine the transcription start site. In **figure 3.7** it can be seen that the transcription of the sodorifen cluster initiates at a thymine nucleotide 53 bp upstream of the IPP isomerase start codon (indicated as +1).

As presented above, the sodorifen biosynthesis genes are transcribed as one mRNA molecule. Therefore, it was likely that one promoter, located in the sodorifen cluster upstream sequence, is responsible for the expression of this gene cluster . After successful identification of the transcriptional start site, BPROM online software (Solovyev and Salamov, 2011) was used to search the remaining upstream sequence of *S. ply.* 4Rx13 for a potential promoter. As a result, putative -10 and -35 boxes were identified which almost perfectly coincided with the consensus se-

```
4Rx13       GGTATTTCCTCTTACTGGCTCGCTATAGATCCATCCGATTTGACACCATGACGCAGGCGG   60
HRO-C48     GGTATTTCCTCTTGCTGGCTCGCTATAGATCCATCCGATTTGACACCATGACGCAGGCGG   60
3Re4-18     GGTATTTCCTCTTGCTGGCTCGCTATAGATCCATCCGATTTGACACCATGACGCAGGCGG   60
S13         GGTATTTCCTCTTGCTGGCTCGCTATAGATCCATCCGATTTGACACCATGACGCAGGCGG   60
V4          GGTATTTCCTCTTGCTGGCTCGCTATAGACCCATCCGATTTGACACCATGACGCAGGCGG   60
            ************* *************** ******************************

4Rx13       GAACAAGAAGTAAATGTTATGTTAACTGCAGCGACGCACAATAACCCCACTCTGCAATCA   120
HRO-C48     GAACAAGAAGTAAATGTTATGTTAACTGCAGCGACGCACAATAACCCCACTCTGCAATCA   120
3Re4-18     GAACAAGAAGTAAATGTTATGTTAACTGCAGCGACGCACAATAACCCCACTCTGCAATCA   120
S13         GAACAAGAAGTAAATGTTATGTTAACTGCAGCGACGCACAATAACCCCACTCTGCAATCA   120
V4          GAACAAGAAGTAAATGTTATGTTAACTGCAGCGACGCACAATAACCCCACACTGCAATCA   120
            ************************************************* *********

4Rx13       ACATGACCGAGACGCTTCATTACGGTCGG-GACAGAGGGTATCGGTGAGCACGTAATATC   179
HRO-C48     ACATGACCGAGACGCTTCATTACGGTCGG-GACAGAGGGTATCGGTGAGCACGTAACATC   179
3Re4-18     ACATGACCGAGACGCTTCATTACGGTCGG-GACAGAGGGTATCGGTGAGCACGTAACATC   179
S13         ACATGACCGAGACGCTTCATTACGGTCGG-GACAGAGGGTATCGGTGAGCACGTAACATC   179
V4          ACATGACCGAGACGCTTCATTACGGTCGGGGACAGAGGGTATCGGTGAGCACGTAACATC   180
            ***************************** ************************** ***

4Rx13       ATTGGGCTAGCCGACCCCTTTGTTTCTGTAGCCGAAAACATCCATCGCCTGGCTTTAACA   239
HRO-C48     ATTGGGCTAGCCGACCCTTTTGTTTCTGTAGCCGAAAACATCCATCGCCTGGCTTTAACA   239
3Re4-18     ATTGGGCTAGCCGACCCTTTTGTTTCTGTAGCCGAAAACATCCATCGCCTGGCTTTAACA   239
S13         ATTGGGCTAGCCGACCCTTTTGTTTCTGTAGCCGAAAACATCCATCGCCTGGCTTTAACA   239
V4          ATTGGACTAGCCGACCCTTTTGTTTCTGTAGCCAAAAATATTCATCACCTGGCTTTGACA   240
            ***** *********** *************** **** ** **** ********* ***

4Rx13       AAAAATAGTTGCACCCTAGCAAAACTCCCACATCGTTGAATTAACATTAAGTTAAACCAT   299
HRO-C48     AAAAATAGTTGCACCCTAGCAAAACTCCCACATCGTTGAATTAACATTAAGTTAAACCAT   299
3Re4-18     AAAAATAGTTGCACCCTAGCAAAACTCCCACATCGTTGAATTAACATTAAGTTAAACCAT   299
S13         AAAAATAGTTGCACCCTAGCAAAACTCCCACATCGTTGAATTAACATTAAGTTAAACCAT   299
V4          TAAAATAGTTTCGCCATAGCAAAACTCCCACATCGTTGAATTAACATTAAGTTAAACCAT   300
             ********* * ** ********************************************

4Rx13       AAGTTAAAAATATATCACTCGCTGATACTTCATCATAAAATAGTCATCATCATGATTAAT   359
HRO-C48     AAGTTAAAAATATATCACCAGCTGATACTTCATCATAAAATAGTCATCATCATGATTAAT   359
3Re4-18     AAGTTAAAAATATATCACCAGCTGATACTTCATCATAAAATAGTCATCATCATGATTAAT   359
S13         AAGTTAAAAATATATCACCAGCTGATACTTCATCATAAAATAGTCATCATCATGATTAAT   359
V4          AAGTTAAAAATATATCACCAGCTGATACTTCATCATAAAATAGTCATCATCATGATTAAT   360
            ******************  ****************************************

4Rx13       TTATTTTTCTTAAAAACCGTCATTAAATAAAATTTGACAATGAAAGAAATTCGTCTTTAA   419
HRO-C48     TTATTTTTATTAAAAGCCATCATTAAATAAAATTTGACAATGAAAGAAATGCGTCTTTAA   419
3Re4-18     TTATTTTTATTAAAAGCCATCATTAAATAAAATTTGACAATGAAAGAAATGCGTCTTTAA   419
S13         TTATTTTTATTAAAAGCCATCATTAAATAAAATTTGACAATGAAAGAAATGCGTCTTTAA   419
V4          TTATTTTTCTTAAAAACCATCATTAAATAAAGTTTGACAATGAAAAAAATGCGTCTTTAA   420
            ******** ****** ** ************ ************* **** *********

4Rx13       TTAAATTTCATTGATTAAAAAACACCCAATGCTCAATCGAATTTATTTCGGGGGTCACTC   479
HRO-C48     TTAAATTTCATTGATCAAAAAACACCCAATGCTCAATCGAATTTATTTCGGGGGTCACTC   479
3Re4-18     TTAAATTTCATTGATCAAAAAACACCCAATGCTCAATCGAATTTATTTCGGGGGTCACTC   479
S13         TTAAATTTCATTGATCAAAAAACACCCAATGCTCAATCGAATTTATTTCGGGGGTCACTC   479
V4          TTAAATTTCATTGATCAAAAAACACCCTATCTGCAATCGAATTTATTTCGGGGGTCACTC   480
            *************** *********** **   ****************************

4Rx13       G---------      480
HRO-C48     GTGGAAGAA       488
3Re4-18     ---------       479
S13         GTGGAAGAA       488
V4          ---------       480
```

Figure 3.5: Alignment of the sodorifen cluster upstream sequence in the different sodorifen producer strains. Alignment of the sodorifen cluster upstream sequence in the producing isolates *S. ply.* 4Rx13, HRO-C48, 3Re4-18, S13 and V4 was performed with the Clustal Omega online software (McWilliam *et al.*, 2013). Asterisks indicate matches, dashes and red nucleotides correspond to mismatches.

```
4Rx13      GGTATTTCCTCTTACTGGCTCGCTATAGATCCATCCGATTTGACACCATGACGCAGGCGG    60
AS9        GGTTTTTCCTCTTGCTGGCTCGCTATCTACCTATCCGATTTGACACCATGACGCAGGCGG    60
           *** ********* ************  * * ****************************

4Rx13      GAACAAGAAGTAAATGTTATGTTAACTGCAGCGACGCACAATAACCCCACTCTGCAATCA    120
AS9        GAACAAGAAGTAAATGTTATGTTAACTGCAGCGACGCACAATAACCCCACTCTTCAATCA    120
           ************************************************** ******

4Rx13      ACATGACCGAGACGCTTCATTACGGTCGGGACAGAGGGTATCGGTGAGCACGTAATATCA    180
AS9        ACATGACCGAGACGCTTCACTACAGTCCGGGACAGAG-----------GGTTTAGCATCA    169
           ****************** *** *** **      *              **  ****

4Rx13      TTGGGCTAGCCGACCCCTTTGTTTCTGTAGCCGAAAACATCCATCGCCTGGCTTTAACAA    240
AS9        TTGGGCTAGCCGACCCTTTTGTTTCTGTAGCTAAAATCATTCATCATCTGGCTCTGACAT    229
           ********************************  *** *** ****  ****** * ***

4Rx13      AAAATAGTTGCACCCTAGCAAAACTCCCACATCGTTGAATTAACATTAAGTTAAACCATA    300
AS9        AAAATATATTTCGCCACGGA-ATCCCTCTCACCATTGAATTAACCTTGAACTCATCCATA    288
           ******  *    **  * * * * * * ** * ********** ** *  * * *****

4Rx13      AGTTAAAAATATATCACTCGCTGATACTTCATCATAAAATAGTCATCATCATGATTAATT    360
AS9        AGTAAAAAATATGTCACCAACTTATACATCATCG--------------------TTATTA    328
           *** ******** ****   ** **** *****                     *** *

4Rx13      TATTTTTCTTAAAAACCGTCATTAAATAAAATTTGACAATGAAAGAAATTCGTCTTTAAT    420
AS9        ATTTATTTTTTCAAACCCATCATTAAATAAATTTGACAATGAAAGAAATGCGTCTTTAAT    388
             ** ** **  *****     * **  ******************** **********

4Rx13      TAAATTTCATTGATTAAAAAACACCCAATGCTCAATCGAATTTATTTCGGGGGTCACTCG    480
AS9        TAAATTTCATTGATCAAAAAACATACTTCCTGCAATCGAATTTATTTCGGGGGTCACTCG    448
           ************** ********  *      ****************************

4Rx13      --------     480
AS9        TGGAAGAA     456
```

Figure 3.6: Alignment of the sodorifen cluster upstream sequence in *S. ply.* 4Rx13 and AS9. Alignment of the sodorifen cluster upstream sequence in the sodorifen producing isolate *S. ply.* 4Rx13 with the non-producer *S. ply.* AS9 was performed with the Clustal Omega online software (McWilliam *et al.*, 2013). Asterisks indicate matches, dashes and red nucleotides correspond to mismatches.

quences for bacterial σ^{70} dependent promoters (-10=TATAAT; -35=TTGACA; figure 3.7). Only one nucleotide exchange was identified in the -10 box in *S.p.* 4Rx13. Interestingly, also σ^{S} dependent transcription of the sodorifen cluster would be possible since the -10 and -35 box consensus sequences of the σ^{S} factor are identical to the ones for σ^{70}.

3.1.5 Role of the σ^{S} factor in expression of the sodorifen cluster genes

Analysis of the promoter region revealed two sigma factors which are potentially involved in transcription of the sodorifen cluster in *S.p.* 4Rx13, σ^{S} and σ^{70}. To first assess the role of σ^{S} in expression of the cluster genes, insertional deletion mutagenesis was performed on the *rpo*S gene, which encodes for the sigma factor S. By insertion of a functional cassette, disruption of the open reading frame of *rpo*S was achieved and, at the same time, a kanamycin resistance gene was integrated into the *S.p.* 4Rx13 genome allowing selection of positive mutants by addition of the

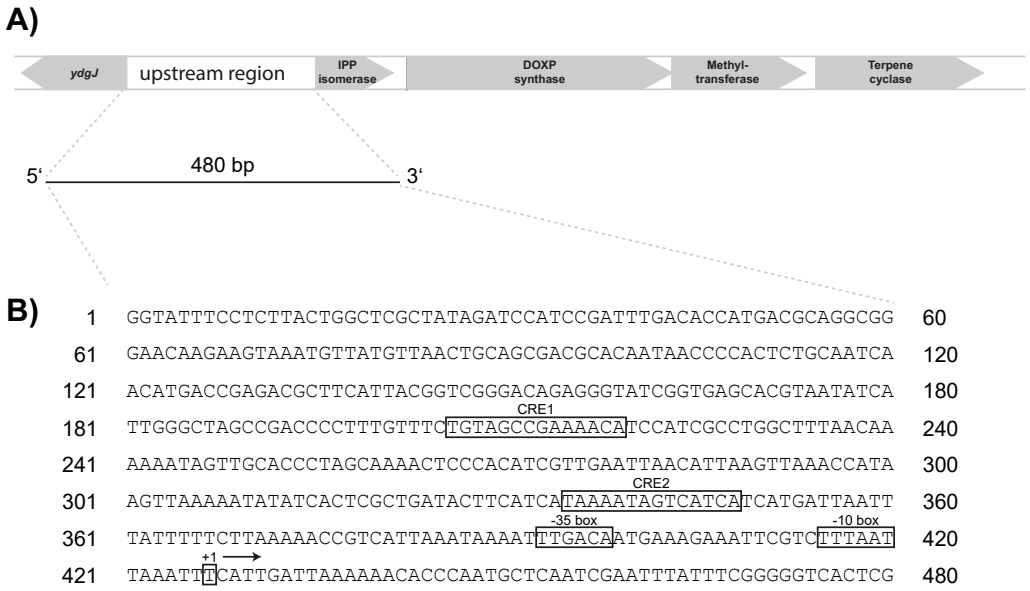

Figure 3.7: Identification of the transcription start site and the promoter region of the sodorifen cluster in *S. ply.* 4Rx13. A) Location and size of the sodorifen cluster upstream sequence in the genome of *S. ply.* 4Rx13. **B)** Sodorifen cluster upstream sequence. The transcription start site, determined by 5'-RACE-PCR, is indicated at position +1. -10 and -35 regions represent the potential promoter region of the sodorifen cluster. CRE1 and CRE2 refer to potential binding sites of the cAMP/CRP complex (for details see section 3.2.3).

respective antibiotic. The obtained mutant was first analyzed for correct insertion of the functional cassette by PCR. In **figure 3.8 A** it can be seen that in the wild type strain of *S. ply.* 4Rx13 only amplification of the *rpo*S gene itself was possible (lane 1), whereas for the mutant strain *rpo*S::Km also reactions using insert-specific primers resulted in amplification products of the expected sizes (lanes 6-8). Furthermore, the amplification product using gene-specific primers showed a 1.6 kb larger size in *rpo*S::Km than in the WT, proving insertion of the functional cassette. Also, combinations of gene- and insert-specific primers resulted in amplification products of the expected sizes, verifying correct insertion of the resistance cassette. Secondly, stable maintenance of the insertion was proved by PCR after 24 h, 48 h and 72 h cultivation in minimal medium supplemented with 55 mM succinate using gene-specific primers (**figure 3.8 C**). Thirdly, growth behaviour of the *rpo*S mutant strain was compared to *S. ply.* 4Rx13 wild type in MM + succinate by measuring the living cell number (CFU/ml; **figure 3.8 B**). During the lag-phase, no differences in growth were observable. But interestingly, after 24 h cultivation *rpo*S::Km showed a higher amount of living cells in comparison to the WT. After this time point, living cells of the *rpo*S mutant strain started to decrease, indicating dying of the culture whereas the living cell number in the wild type culture remained constant until 72 h. Finally,

the volatile emission of *rpo*S::Km was examined and compared to the volatile spectrum of *S. ply.* 4Rx13 wild type (**figure 3.8 D**). Thereby it became obvious that the mutant strain deficient for the σ^S factor was no longer capable to produce sodorifen, indicating its crucial role for the sodorifen cluster expression.

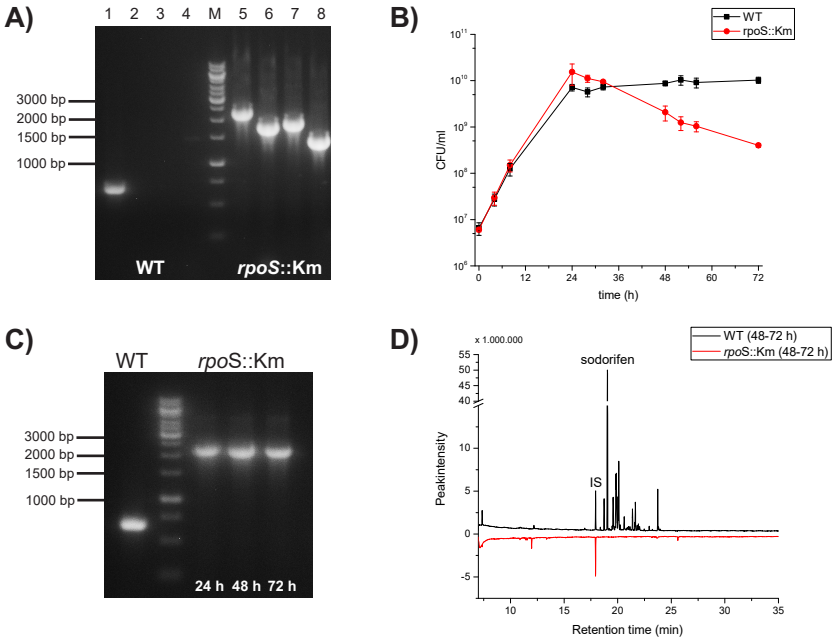

Figure 3.8: Characterization of the *rpo*S::Km insertion mutant of *S. ply.* 4Rx13. A) Verification of correct insertion of the functional cassette by PCR. In lanes 1-4 results for the *S. ply.* 4Rx13 wildtype are shown and in lanes 5-8 for the *rpo*S mutant. Lanes 1 & 5 represent amplification products with gene-specific primers for the *rpo*S gene (665 bp for the WT; 2.3 kb for *rpo*S::Km). Lanes 4 & 8 depict amplification products of primer specific for the functional cassette (1.4 kb). Lanes 2 & 6 show PCR products after amplification with gene- and cassette-specific primers (1.8 kb; left border),as well as lanes 3 & 7 (right border; 1.9 kb). M = marker (GeneRuler® 1 kb, Thermo Fisher Scientific, Madison, USA). **B)** Growth comparison of *S. ply.* 4Rx13 WT and *rpo*S::Km in minimal medium supplemented with 55 mM succinate. The living cell number (CFU/ml) was determined periodically for up to 72 h. Error bars represent standard deviation (n=3). **C)** Stability of the mutation of *rpo*S in *S. ply.* 4Rx13 was determined in 24 h intervals for up to 72 h of cultivation in MM + succinate using PCR. **D)** Volatile spectrum of *S. ply.* 4Rx13 wildtype (black) in comparison to the *rpo*S mutant (red) after 72 h of cultivation in MM + succinate. Volatiles were sampled using the VOC collection system and analyzed by GC/MS. IS = internal standard (nonylacetate, 5 ng).

This hypothesis was further strengthened by performing comparative expression analyses of the sodorifen cluster between *S. ply.* 4Rx13 wild type and *rpo*S::Km (**figure 3.9**). Using Northern blot technique it became obvious that in *rpo*S::Km expression of the terpene cyclase gene was not detectable anymore in contrast to the wild type strain. Even after doubling the amount of RNA used in the Northern blot (figure 3.9 B) no expression of the terpene cyclase gene could be observed. These findings further solidify the hypothesis that sodorifen cluster expression is strongly

dependent on the σ^S factor in *S. ply.* 4Rx13.

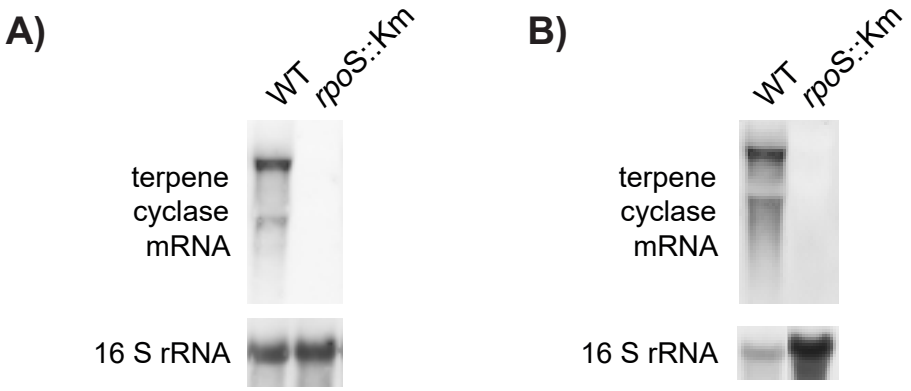

Figure 3.9: Expression of the terpene cyclase in *S. ply.* 4Rx13 *rpo*S::Km in comparison to the wild type. *S. ply.* 4Rx13 WT and *rpo*S::Km were cultivated for 24 h in minimal medium supplemented with 55 mM succinate. In **A)** 5 µg of WT and mutant RNA were used for Northern blot, whereas in **B)** the amount of mutant RNA was doubled (10 µg). Terpene cyclase mRNA (upper panel) was hybridized with a DIG-labelled probe and finally detected using fluorescence measurement for 1 min. As a positive control the 16 S rRNA (lower panel) was visualized in the same manner.

3.1.6 Influence of abiotic stress on the sodorifen emission in *Serratia plymuthica* 4Rx13

As presented above, the stationary phase sigma factor σ^S is responsible for the strong expression of the sodorifen biosynthesis cluster in *S. ply.* 4Rx13. Additionally, σ^S is also involved in the reactions of bacteria to different abiotic stresses, e.g. pH shift, osmotic stress and heat shock, making a function for sodorifen in stress compensation possible. Therefore, the influence of different stress factors (pH, salt stress, heat shock) on the sodorifen emission in *S. ply.* 4Rx13 was assessed. In all experiments minimal medium supplemented with 55 mM succinate (pH 6.2) was used as a reference. Concerning the effect of pH on the sodorifen emission, the MM was adjusted to acidic (5.2, 5.5) as well as neutral (6.2, 7.0) and alkaline pH (8.0). Furthermore, the reaction of *S. ply.* 4Rx13 to osmotic stress was investigated by addition of 1-2 % sodium chloride to the MM. At last, growing cultures of *S. ply.* 4Rx13 were subjected to a transient heat shock at 42 °C for 30 min after 5 h of cultivation. The effects of the different stress conditions on the sodorifen emission were determined using the VOC collection system with subsequent GC/MS analysis of the gained eluates. Finally, the detected sodorifen amounts were quantified using an internal standard (5 ng nonylacetate) and the living cell number of the respective cultures.

In **figure 3.10 A** it can be seen that both, shifting the pH value in the acidic (pH

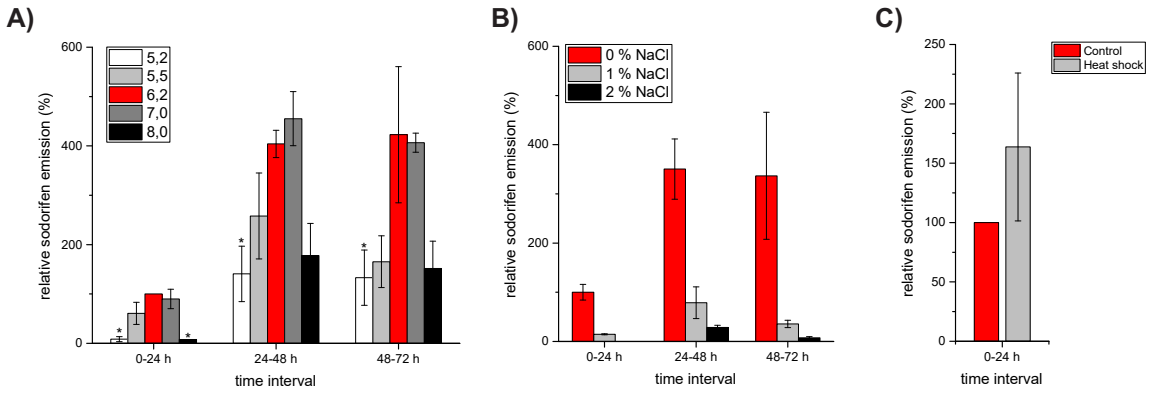

Figure 3.10: Effect of abiotic stress on the sodorifen emission in *S. ply.* 4Rx13. *S. ply.* 4Rx13 was cultivated in minimal medium supplemented with 55 mM succinate (pH 6.2). **A)** Effect of pH on sodorifen emission in *S. ply.* 4Rx13. pH values were adjusted to 5.2, 5.5, 6.2, 7.0 and 8.0. **B)** Influence of osmotic stress on the sodorifen emission in *S. ply.* 4Rx13. 1-2 % NaCl were added to MM + succinate. **C)** Heat shock was applied to a 5 h grown culture of *S. ply.* 4Rx13 at 42 °C for 30 min. Subsequently, the culture was cultivated at 30 °C until 24 h post-inoculation. In all cases, sodorifen emission after 24 h cultivation in MM + succinate (pH 6.2) was used as a control (100 %; red bars). Emitted volatiles were analyzed using the VOC collection system and GC/MS. Sodorifen emission was quantified with an internal standard (nonylacetate, 5 ng) and calculated relative to the living cell number present in the cultures. For this purpose, the culture with pH 6.2 (0-24 h) was used as a reference (100 %; red bars). Error bars represent standard deviations (n $\geq$ 3) or deviations from the mean value (* n=2).

5.2 & 5.5) and alkaline (pH 8.0) range, resulted in a strong reduction of the sodorifen emission in *S. ply.* 4Rx13 in comparison to growth in neutral medium (pH 6.2). In detail, sodorifen emission was decreased at pH 5.2 by 65-92 %. At pH 5.5 less severe decrease of sodorifen was found with 36-61 %. Raising the pH value to 8.0 caused an inhibition of sodorifen emission by about 55-92 % which is comparable to the values obtained in acidic medium (pH 5.2). In contrast to this, a slight increase of the pH from 6.2 to 7.0 showed no significant effect on the sodorifen emission in *S. ply.* 4Rx13.

Induction of hyperosmotic stress in *S. ply.* 4Rx13 by addition of sodium chloride, lead to a significant decrease in sodorifen emission (**figure 3.10 B**). In quantitative terms this means, during cultivation in MM + 1 % NaCl the detected sodorifen amount per cell reduced by 77-89 % in comparison to medium without sodium chloride. The most severe effect was found after addition of 2 % NaCl to the culture medium. Here, after 24 h cultivation no sodorifen was detectable. Only upon further incubation for 48 h and 72 h, slight sodorifen emission was found with 8 % and 2 %, respectively, in comparison to the control culture without osmotic stress. Besides two permanent stress conditions (pH and salt), also a transient stress situation was tested for its effect on sodorifen emission, namely heat shock. Raising the growth temperature of

S. ply. 4Rx13 for only 30 min from 30 °C to 42 °C provoked an increase of sodorifen emission by about 63 % in comparison to constant growth at 30 °C (**figure 3.10 C**). In conclusion, inducing permanent stress, which affects the living cell number of *S. ply.* 4Rx13, caused significant decreases in sodorifen emission. In contrast to this, transient stress lead to a considerable surge of sodorifen emission.

3.2 Regulation of sodorifen emission by carbon catabolite repression

Previous studies were concerned with the dependence of the sodorifen amount being emitted on the carbon source present in the medium (Weise, 2013). As a result, the sodorifen emission of *S. ply.* 4Rx13 was about 20-fold higher during cultivation in minimal medium supplemented with 55 mM succinate (MM + succinate) than in complex medium (Nutrient Broth). Contrary to this, the relative sodorifen emission decreased to almost zero when glucose was used as the sole carbon source (MM + glucose; **figure 3.11 A)**. Interestingly, the growth of *S. ply.* 4Rx13 was even better in MM + glucose in comparison to MM + succinate, which excludes a decrease in cell number to be responsible for this effect (**figure 3.12 A**). *S. ply.* 4Rx13 reached higher living cell numbers in MM + glucose. Furthermore, growth parameters determined in both media, reflecting the cell mass (growth rate, doubling time, **figure 3.12 B**) and cell density (division rate, generation time, **figure 3.12 D**), clearly showed that bacterial growth of *S. ply.* 4Rx13 was impaired in MM + succinate in comparison to growth on glucose. Especially the growth rate which resembles the number of cell mass doublings per hour was reduced in MM + succinate by more than 50 % in comparison to MM + glucose. Likewise, the duration of cell mass doubling was increased in succinate containing medium to 2.25 h in contrast to MM + glucose where the doubling time was less than 1 h. Also, cell density dependent parameters were found to be reduced in MM + succinate in comparison to MM + glucose. The division rate (duplications of cell number per hour) declined from 0.9 h^{-1} in MM + glucose to 0.71 h^{-1} in MM + succinate. Furthermore, the time needed for doubling the cell number was increased in succinate containing medium by ca. 30 min in comparison to growth in MM + glucose.

To assess a possible correlation between the sodorifen emission and the expression of the sodorifen biosynthesis genes, first, RT-PCR was performed. Therefore, RNA was isolated from *S. ply.* 4Rx13 after 24 h cultivation in minimial medium supplemented either with 55 mM succinate or glucose. From each sample 6 µg of RNA were used for subsequent DNaseI digestion to remove residual DNA. Afterwards, 1 µg RNA was used for each RT-PCR reaction. The results are presented in **fig-**

ure 3.11 B. Lane 1 represents the negative control (no reverse transcriptase used) and proves that there was no contamination of the RNA isolates with DNA. Furthermore, lane 6 depicts the amplification products of the glyceraldehyde 3-phosphate dehydrogenase as a reference for equal RNA load. Lanes 2 to 5 represent the amplification products of the sodorifen cluster genes, starting with the terpene cyclase (963 bp), followed by the methyltransferase (887 bp), DOXP synthase (1707 bp) and the IPP isomerase (258 bp). As can be seen in figure 3.11 B, it was possible to amplify the mRNA of the sodorifen cluster genes after cultivation in MM+succinate as well as in MM + glucose. Interestingly, the signals for the sodorifen cluster genes are stronger after cultivation in succinate-containing medium than in MM + glucose, despite an almost equal signal intensity in the positive control. This becomes most obvious when looking at the amplification product of the DOXP synthase (lane 4) which was detectable under succinate-containing conditions but not in MM + glucose, most likely due to an expression level below the detection limit of this method. Secondly, the transcription level of the sodorifen cluster genes was quantified using Northern blot and the AIDA Image Analyzer software (v.4.50, raytest, Straubenhardt, DE). For this purpose, the fluorescence value of the terpene cyclase after 24 h cultivation in MM + succinate was set to 100 % and the fluorescence in MM + glucose was calculated relative to it. As can be seen in **figure 3.11 C** the fluorescence level of the positive control (16 S rRNA, lower panel) is equal, whereas for the terpene cyclase it is significantly higher after cultivation in MM + succinate than in MM + glucose. Quantification revealed that the transcription rate of the terpene cyclase in MM + glucose amounts to 5 % and is therefore 20-fold lower than in MM + succinate.

Furthermore, it was tested whether the effects of succinate and glucose are also applicable to the other sodorifen producers (**figure 3.11 D**). As a result, glucose inhibited the sodorifen emission completely in *S. ply.* HRO-C48, 3Re4-18, S13 and V4 (results not shown), whereas succinate showed a stimulating effect on the sodorifen emission in these strains similar to *S. ply.* 4Rx13. Surprisingly, the increase in the sodorifen emission was even more pronounced in the other producer strains, ranging from 5.8-fold in *S. ply.* HRO-C48 (24-48 h) to over 200-fold increase in *S. ply.* Re4-18 (48-72 h). The only exception was observed for the sodorifen producing strain *S. ply.* V4, which was not capable of growing in MM + succinate for longer than 24 h, whereas growth in glucose-containing medium was comparable to *S. ply.* 4Rx13 (results not shown).

Finally, the effect of increasing glucose concentrations on sodorifen emission in *S. ply.* 4Rx13 was analyzed (**figure 3.11 E**). As a result, emission levels in complex medium supplemented with glucose were decreased up to 100 % with the extent of repression being dependent on glucose concentrations. Thus, 10 mM

glucose yielded the lowest sodorifen inhibition (45 % - 91 %) and 100 mM the highest (88 % - 100 %). Moreover, it became obvious that with increasing cultivation time also sodorifen emission increased in the respective culture which was probably due to glucose consumption. In comparison to this, different amounts of glucose were also added to minimal instead of complex medium. In this case, inhibition of sodorifen emission was even more pronounced ranging from 98 % - 100 %. Also, repression was lowest using the smallest glucose concentration (10 mM), although differences to higher amounts were less pronounced than in complex medium.

In conclusion, the sodorifen emission was found to be significantly reduced under glucose-containing conditions, whereas addition of succinate lead to a marked increase. These differences could be attributed to variations in the transcription level of the sodorifen cluster genes and gave rise to the hypothesis that sodorifen biosynthesis is controlled by carbon catabolite repression.

3.2.1 Effect of mixed carbon cultures on *S. ply.* 4Rx13

In the past, experiments were performed investigating the growth and sodorifen emission of *S. ply.* 4Rx13 in media containing several carbon sources. Either glucose was added to minimal medium supplemented with succinate during stationary phase or vice versa (late addition of succinate to MM + glucose) (Weise, Domik & Piechulla; unpublished data). Late addition of glucose to MM + succinate caused a stop of sodorifen emission, whereas addition of succinate to glucose containing MM did not induce production of this volatile. These findings further substantiated the assumption of involvement of carbon catabolite repression in the production of sodorifen.

3.2.1.1 Growth and glucose consumption in mixed carbon cultures

The mechanism of carbon catabolite repression often goes along with diauxic growth of the bacteria in mixed carbon cultures, i.e. media containing two or more distinct carbon sources (Epstein *et al.*, 1966; Monod, 1978). Therefore, growth analysis of *S. ply.* 4Rx13 was performed in minimal medium containing only 55 mM glucose and minimal medium containing both, glucose and succinate, each with a concentration of 55 mM to ensure equal glucose content. During the exponential growth phase (first 14 h of cultivation), the living cell number (2.2.3.2) as well as the glucose content (2.2.3.4) of each culture were determined in a 2 h interval. Finally, the last samples were taken during stationary phase (after 24 h for growth experiments; after 48 h for glucose assays). Also, glucose consumption in both, MM + glucose and + glucose & succinate was calculated by setting the glucose concentration at 0 h to

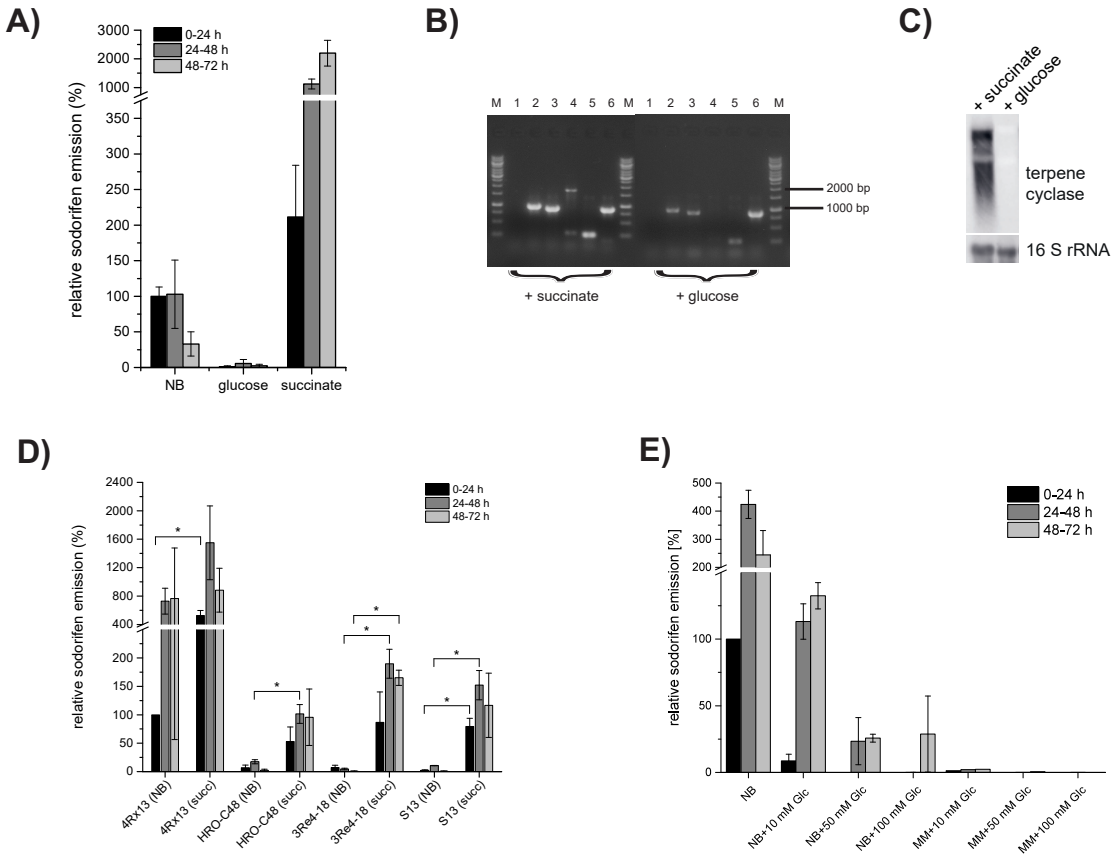

Figure 3.11: Sodorifen emission and cluster expression depending on the carbon source. *S. ply.* 4Rx13 was cultivated in complex medium (NB) or minimal medium supplemented with either 55 mM succinate or glucose. **A)** Relative sodorifen emission. The volatiles were collected every 24 h by solid phase micro extraction (SPME) and analyzed using GCMS. The peak area of sodorifen after 24 h of cultivation in complex medium was used as a reference and set to 100 %. Experiments were performed in triplicates. Error bars represent the standard deviation. Modified from Weise (2013). **B)** RT-PCR of the sodorifen cluster genes. Lane 1 = negative control (no reverse transcriptase), lane 2=terpene cyclase, lane 3 = methyltransferase, lane 4 = DOXP synthase, lane 5 = IPP isomerase, lane 6 = positive control (GAP-DH). M= marker (GeneRuler 1 kb, Thermo Fisher Scientific, Madison, USA). **C)** Northern blot of the terpene cyclase. In each lane 5 µg of total RNA were applied. A DIG-dUTP-labelled probe was used for detection of terpene cyclase mRNA (upper panel). A second hybridization with a 16 S rRNA probe was used as a positive control (lower panel). Hybridization of probes to the corresponding mRNAs was detected by fluorescence measurement for 1 min. RNA isolation for B and C was performed after 24 h of cultivation in the respective medium. **D)** Relative sodorifen emission of *S. ply.* 4Rx13, HRO-C48, 3Re4-18 and S13 in complex medium (NB) and MM + succinate (succ). **E)** Relative sodorifen emission of *S. ply.* 4Rx13 during cultivation either in complex medium (NB) or minimal medium (MM) supplemented with 10 mM, 50 mM and 100 mM glucose. For E) and F) volatiles were trapped with the closed VOC collection system (modified after Kai *et al.* (2010)) and analyzed by GCMS. The peakarea of sodorifen after 24 h cultivation in complex medium by *S. ply.* 4Rx13 was used as a reference and set to 100 %. Experiments were performed in triplicates. Error bars represent the standard deviation (n=3). * $p \leq 0.05$.

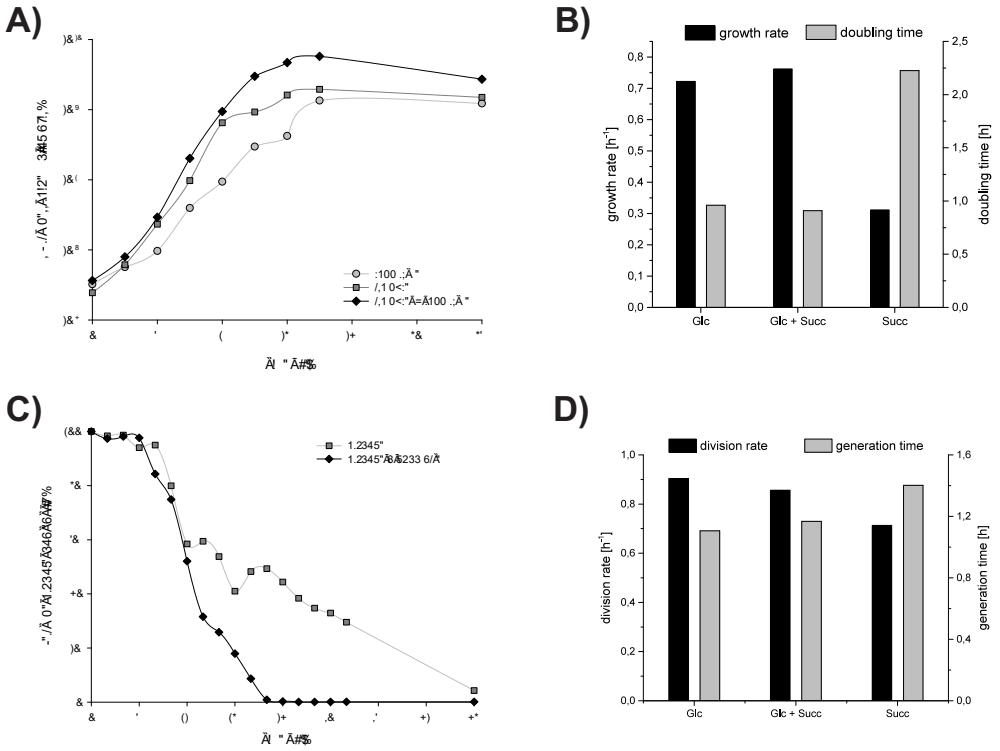

Figure 3.12: Growth and glucose consumption of *S. ply.* 4Rx13 in different carbon sources. *S. ply.* 4Rx13 was cultivated in minimal medium supplemented with 55 mM succinate, 55 mM glucose or a mixture of both (55 mM each). **A)** Growth of *S. ply.* 4Rx13 in MM + succinate (circles), glucose (squares) or glucose + succinate (diamonds, each 55 mM). Growth was determined by measuring the living cell number (CFU/ml) in regular time intervals until 24 h. **B)** Cell mass dependent growth parameters, growth rate (black bars) and doubling time (grey bars). **C)** Glucose consumption of *S. ply.* 4Rx13 during cultivation in MM + 55 mM glucose or + 55 mM glucose and succinate each. Relative glucose content was calculated with the initial glucose concentration used as a reference (100 %). **D)** Cell density dependent growth parameters, division rate (black bars) and generation time (grey bars). For B) and D) Glc = MM + 55 mM glucose; Glc+Succ = MM + 55 mM glucose & succinate each; Succ = MM + 55 mM succinate.

100 % and monitoring its decrease throughout growth. **Figure 3.12 C** presents the glucose consumption of *S. ply.* 4Rx13 in the pure carbon culture (+ 55 mM glucose) in comparison to the mixed carbon culture (each 55 mM glucose & succinate).

In each case, a short lag phase (0-2 h) was followed by an exponential growth phase which merged into the stationary phase after 10 h cultivation. But indeed, in the mixed carbon culture the living cell number reached up to 3-times higher values in the stationary phase than in the pure glucose culture. Surprisingly, there is no difference to be seen in the course of the growth curves between mixed and pure carbon culture, indicating that *S. ply.* 4Rx13 does not perform diauxic growth in minimal medium supplemented with a mixture of glucose and succinate. Comparison of cell mass and cell density dependent growth parameters (**figure 3.12 B and D**), revealed no marked differences between growth of *S. ply.* 4Rx13 in MM + glucose or

MM + glucose and succinate. Concerning the glucose consumption of *S. ply.* 4Rx13 in both cultures, it became obvious that the decline in glucose concentration was more pronounced in the mixed carbon culture than in the pure glucose culture.

During the first 6 h of cultivation there is almost no decrease in glucose content visible in both cultures. Only after 6 h, in the mixed carbon culture, and 8 h, in the pure glucose culture, glucose is starting to be consumed by *S. ply.* 4Rx13 yielding a value of about 7 % per hour. In the mixed carbon culture glucose consumption stays constant with a rate of ca. 6 % per hour resulting in complete removal of glucose from the medium after 22 h cultivation. In contrast to this, in the pure glucose culture, consumption rate decreases after 10 h cultivation from 7.5 %/h to only 2.1 %/h. Furthermore, complete removal of glucose took 48 h in MM + 55 mM glucose.

3.2.1.2 Sodorifen emission in mixed carbon cultures

Additional to measuring the glucose consumption in mixed carbon cultures, also the sodorifen emission was determined. For this purpose, *S. ply.* 4Rx13 was cultivated in either complex medium (NB) or minimal medium supplemented with a mixture of glucose and succinate (each 55 mM). Volatiles were collected in 24 h time intervals with the VOC collection system (2.4.1) and subsequent GCMS analysis (2.4.2). Additionally, the living cell number was determined (2.2.3.2) and related to the amount of emitted sodorifen (relative sodorifen emission). The emission level of *S. ply.* 4Rx13 after 24 h of cultivation in complex medium was used as a reference (100 %). As a result, addition of glucose to MM + succinate decreased the sodorifen emission to values of about 0-9 % in comparison to complex medium, with a steady increase in sodorifen emission being observed with longer cultivation time (**figure 3.13**). Therefore, values of sodorifen emission obtained in mixed carbon sources are almost equal to cultivation in pure glucose medium (figure 3.11 A), which is why glucose is thought to act as a repressor on the sodorifen emission rather than succinate as an activator.

3.2.2 Role of the central CCR genes *cya* and *crp* in the regulation of the sodorifen emission

Accumulating evidence for regulation of the sodorifen emission by carbon catabolite repression (CCR) made the investigation of central CCR genes necessary. Therefore, the genes encoding for the adenylate cyclase (*cya*) and for the cAMP receptor protein (*crp*) in *S. ply.* 4Rx13 were subjected to mutagenesis by insertional deletion. Integration of a functional cassette encoding for a kanamycin resistance gene, was performed by homologous recombination. For this purpose, 50 bp complemen-

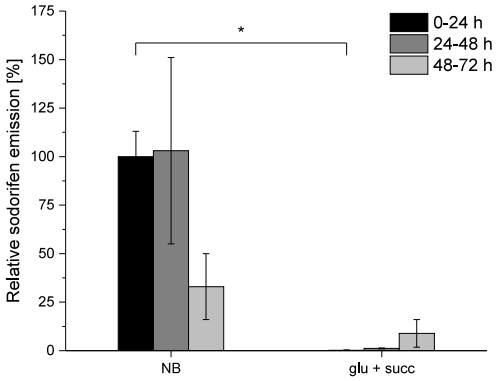

Figure 3.13: Relative sodorifen emission of *S. ply.* 4Rx13 during cultivation in mixed carbon cultures. Sodorifen emission of *S. ply.* 4Rx13 was determined during cultivation in complex medium (NB) or minimal medium + succinate and glucose (each 55 mM; glu+succ) using the VOC collection system and subsequent GCMS analysis. Sodorifen emission was calculated relative to the living cell number and with the emission level in NB after 24 h cultivation as a reference (100 %). Error bars indicate standard deviation (n=3). * $p < 0.01$.

tary to the gene of interest were attached to the functional cassette by PCR. After electrotransformation of the resulting product in *S. ply.* 4Rx13, insertion of the construct into the bacterial genome took place. To support the process of homologous recombination, a plasmid (pRed/ET) was introduced into *S. ply.* 4Rx13 previous to transformation with the functional cassette. The resulting mutants defective in cAMP production (*cya*::Km) and binding (*crp*::Km) were subsequently analyzed.

3.2.2.1 Analysis of the *cya*::Km insertion mutant

At first, the obtained mutant was checked for the correct insertion of the functional cassette by polymerase chain reaction. Therefore, primer combinations specific for the adenylate cyclase gene, for the functional cassette, as well as combinations of them were used. In **figure 3.14 A** it can be seen that for the wild type (lanes 1-4) only one amplification product with the gene-specific primers could be obtained corresponding to the expected size of 1106 bp (lane 1). In contrast to this, for the *cya* mutant (lanes 5-8) the gene-specific primers revealed an amplification product of about 2.7 kb in size, representing the adenylate cyclase gene (1.1 kb) after insertion of the functional cassette (1.6 kb). Furthermore, also PCR products of expected sizes were obtained with reactions including primers specific for the functional cassette in case of the mutant (lanes 6-8), which were not detectable for the wild type. Additionally, stable maintenance of the mutation in the adenylate cyclase gene throughout cultivation was verified (**figure 3.14 C**). For this purpose, the

cya::Km mutant was cultivated for up to 72 h in complex medium and each 24 h samples were taken and analyzed by colony PCR (2.3.5) using gene-specific primers. As a result, it was possible to obtain an amplification product with ca. 2.7 kb in size at each time point tested leading to the conclusion that the inserted functional cassette was stably maintained throughout growth. After verification of correct insertion of the functional cassette and its stability over time, the growth of the mutant was compared to the wild type to exclude impairment of growth to be responsible for potential differences in VOC emission. Therefore, the living cell number during cultivation in complex medium was monitored for 72 h (**figure 3.14 B**). The results indicate that the insertion in the *cya* gene in *S. ply.* 4Rx13 did not affect the growth behaviour of the mutant in comparison to the wild type. Interestingly, the *cya*::Km mutant was no longer capable of growing in minimal medium containing succinate, probably due to the stimulatory effect of the cAMP/CRP complex on the expression of genes responsible for succinate import into the cell (Kumar, 1976). This is why, investigations of the volatile emission were performed only in complex medium. Finally, comparison of the VOC profiles of both, wild type and *cya*::Km revealed a reduction in the sodorifen emission in the mutant of up to 50 % (**figure 3.14 D**).

To ensure comparability of the sodorifen amounts being emitted by the wild type and the mutant, the relative sodorifen emission was calculated by relating the living cell number of each culture to the peak intensity of sodorifen measured by GC/MS (**figure 3.15**). The results revealed that in the wild type, as well as in the *cya*::Km insertion mutant, the sodorifen emission is increasing during growth in complex medium until reaching its maximum at 24-48 h and is afterwards decreasing again. Direct comparison of the wild type emission levels to those reached by the *cya* mutant strain showed that in the time interval of 0-24 h the relative sodorifen emission of the mutant exceeds that of the wild type by about 40 %. From 24-48 h the sodorifen emission in *cya*::Km is reduced by 80 % in comparison to the wild type. This decrease is further enhanced during 48-72 h of growth, where the wild type emits about 112 % more sodorifen than the *cya*-negative mutant. In general, a tendency towards reduced sodorifen emission can be observed upon insertional deletion mutagenesis of the adenylate cyclase gene in *S.ply.* 4Rx13.

3.2.2.2 Analysis of the *crp*::Km insertion mutant

For the *crp*::Km insertion mutant, again, first the genotype was verified using PCR and primers specific either for the *crp* gene itself, for the inserted functional cassette or combinations of both (**figure 3.16 A**). For the wild type, it was only possible to obtain a fragment with the gene-specific primers having a size of about 550 bp corresponding to the expected size of the unmodified *crp* gene (537 bp). In contrast to

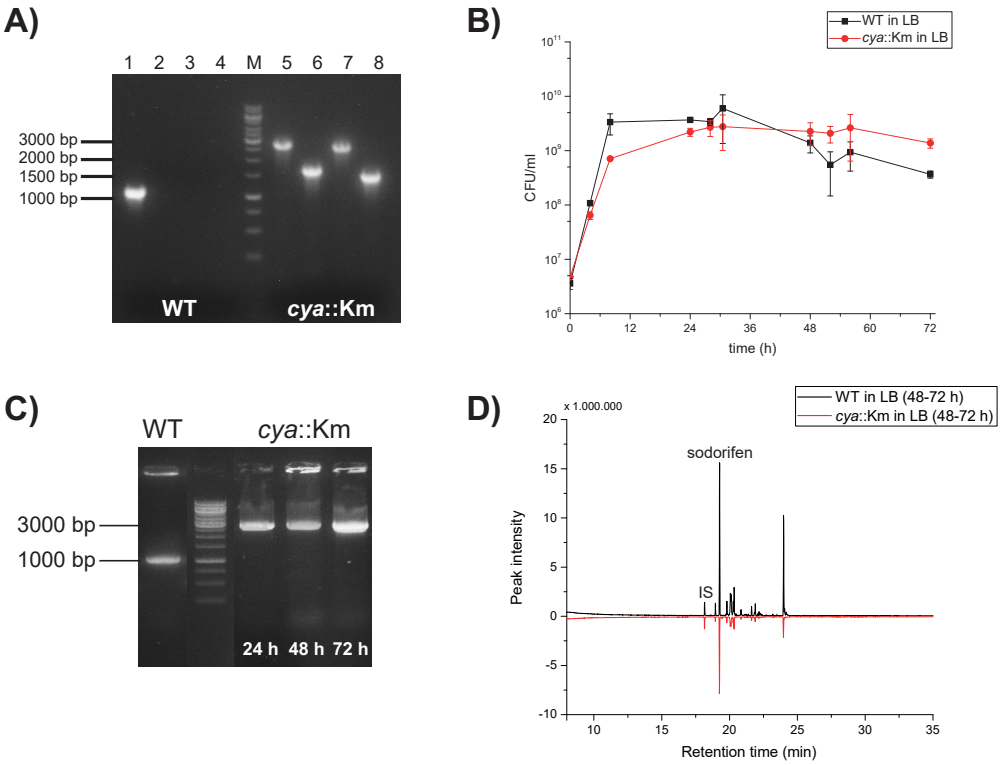

Figure 3.14: Characterization of the *cya*::Km insertion mutant of *S. ply.* 4Rx13. A) Verification of correct insertion of the functional cassette by PCR. In lanes 1-4 results for the *S. ply.* 4Rx13 wildtype are shown and in lanes 5-8 for the *cya* mutant. Lanes 1 & 5 represent amplification products with gene-specific primers for the *cya* gene (1.1 kb for the WT; 2.7 kb for *cya*::Km). Lanes 4 & 8 depict amplification products with primers specific for the functional cassette (1.4 kb). Lanes 2 & 6 show PCR products after amplification with gene- and cassette-specific primers (1.6 kb; left border),as well as lanes 3 & 7 (right border; 2.5 kb). M = marker (GeneRuler® 1 kb, Thermo Fisher Scientific, Madison, USA). **B)** Growth comparison of *S. ply.* 4Rx13 WT and *cya*::Km in complex medium (LB). The living cell number (CFU/ml) was determined periodically for up to 72 h. Error bars represent standard deviation (n=3). **C)** Stability of the mutation of *cya* in *S. ply.* 4Rx13 was determined in 24 h intervals for up to 72 h of cultivation in complex medium (LB) using PCR. **D)** Volatile spectrum of *S. ply.* 4Rx13 wildtype (black) in comparison to the *cya* mutant (red). Volatiles were sampled using the VOC collection system and analyzed by GC/MS. IS = internal standard (nonylacetate, 5 ng).

this, amplification of the *crp* gene in the insertion mutant lead to a fragment matching the expected size of 2.15 kb after insertion of the functional cassette (1.6 kb). Furthermore, it was only possible to obtain amplification products with insert-specific primers when using *crp*::Km DNA as template. The same applies to the combinations of gene- and insert-specific primers. In conclusion, it was possible to verify the correct insertion of the functional cassette and therefore, also the successful disrup-

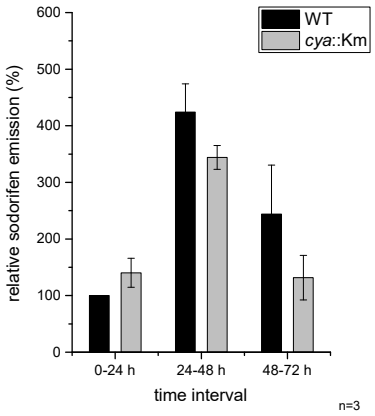

Figure 3.15: Relative sodorifen emission of *cya*::Km in complex medium. *S. ply.* 4Rx13 wild type and *cya*::Km mutant were cultivated for 72 h in complex medium (LB). Volatiles were collected using the VOC collection system in 24 h time intervals. The relative sodorifen emission was calculated in correlation to the living cell number. The sodorifen emission of the wild type from 0-24 h was defined as 100 %. Error bars indicate standard deviation (n=3).

tion of the *crp* gene in *S. ply.* 4Rx13. As can be seen in **figure 3.16 C**, the insertion of the functional cassette was also stably maintained throughout growth in complex medium proved by the enlarged amplification product of the *crp* gene (2.15 kb) gained by PCR with gene-specific primers. Furthermore, the growth of the *crp*::Km mutant in complex medium was monitored for 72 h and compared to the wild type (**figure 3.16 B**). As a result, no significant differences were detectable in the growth behaviour of the mutant compared to the wild type of *S. ply.* 4Rx13. Interestingly, the *crp*::Km mutant was also not able to grow on minimal medium supplemented with succinate anymore, just like *cya*::Km. At last, the volatile emission was investigated upon mutagenesis of the cAMP receptor protein. A direct comparison of the VOC spectra obtained for the wild type and the *crp*::Km mutant, after 72 h cultivation in complex medium, revealed a significant, almost 10-fold, reduction of the sodorifen emission in the mutant compared to the wild type (**figure 3.16 D**).

Finally, the sodorifen amounts being emitted were calculated relative to the living cell number and the value obtained for the wild type at 0-24 h was set to 100 %. From **figure 3.17** it becomes obvious that during the first time interval wild type and *crp*-negative mutant emit almost equal amounts of sodorifen with 100 % and 111 %, respectively. During the 24-48 h interval, the sodorifen emission in the wild type increased to 424 % whereas in the mutant it remained almost constant (125 %). In the last time interval investigated (48-72 h), the wild type showed a decrease in the relative sodorifen emission to 243 % in comparison to the previous interval. The same was applicable to the *crp*::Km mutant which produced only 27 % of the sodorifen amount emitted by the wild type during the first 24 h of growth.

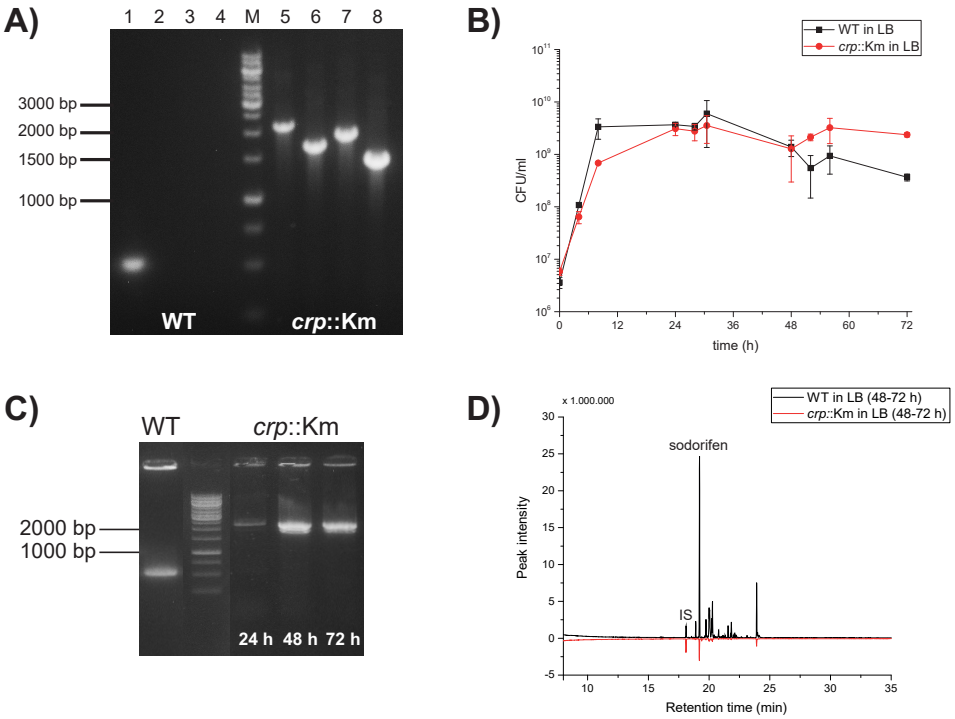

Figure 3.16: Characterization of the *crp*::Km insertion mutant of *S. ply.* 4Rx13. A) Verification of correct insertion of the functional cassette by PCR. In lanes 1-4 results for the *S. ply.* 4Rx13 wildtype are shown and in lanes 5-8 for the *crp* mutant. Lanes 1 & 5 represent amplification products with gene-specific primers for the *crp* gene (537 bp for the WT; 2.15 kb for *crp*::Km). Lanes 4 & 8 depict amplification products with primers specific for the functional cassette (1.4 kb). Lanes 2 & 6 show PCR products after amplification with gene- and cassette-specific primers (1.68 kb; left border),as well as lanes 3 & 7 (right border; 1.89 kb). M = marker (GeneRuler® 1 kb, Thermo Fisher Scientific, Madison, USA). **B)** Growth comparison of *S. ply.* 4Rx13 WT and *crp*::Km in complex medium (LB). The living cell number (CFU/ml) was determined periodically for up to 72 h. Error bars depict standard deviation (n=3). **C)** Stability of the mutation of *crp* in *S. ply.* 4Rx13 was determined in 24 h intervals for up to 72 h of cultivation in complex medium (LB) using PCR. **D)** Volatile spectrum of *S. ply.* 4Rx13 wildtype (black) in comparison to the *crp* mutant (red) during cultivation in complex medium (LB; 48-72 h). Volatiles were sampled using the VOC collection system and analyzed by GC/MS. IS = internal standard (nonylacetate, 5 ng).

3.2.2.3 Influence of *cya* and *crp* on the sodorifen cluster expression

For both, *cya*::Km and *crp*::Km, an overall negative effect on the emission of sodorifen was detectable. One possible reason is a potential role of these two genes concerning the expression strength of the sodorifen cluster genes. To assess this, Northern blot analyses were conducted using RNA isolated from the two mutant

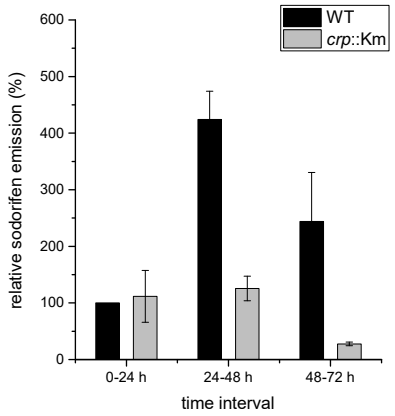

Figure 3.17: Relative sodorifen emission of *crp*::Km in complex medium. *S. ply.* 4Rx13 wild type and *crp*::Km mutant were cultivated for 72 h in complex medium (LB). Volatiles were collected using the VOC collection system in 24 h time intervals. The relative sodorifen emission was calculated in correlation to the living cell number. The sodorifen emission of the wild type from 0-24 h was defined as 100 %. Error bars indicate standard deviations (n=3).

strains and the wild type after 24 h cultivation in complex medium and a DIG-labelled probe specific for the terpene cyclase gene of the sodorifen cluster (**figure 3.18**). As a positive control the 16 S rRNA was detected using the same blot. Quantification of the obtained signals was performed using AIDA Image Analyzer software and by using the wild type expression level as reference. The results indicate that the expression of the terpene cyclase is lowest in the wild type of *S. ply.* 4Rx13 (100 %). In the *crp* and the *cya* insertion mutant the expression level of the terpene cyclase was ca. 3-4 times higher than in the wild type strain (321.55 % and 380.29 %, respectively). The equal 16 S rRNA signals detected, ensured uniform RNA load on the membrane after blotting. In sum, the significant increases in sodorifen cluster expression in *cya*::Km and *crp*::Km explain the increased sodorifen emissions in these two mutant strains in the time interval 0-24 h (figure 3.15, 3.17).

3.2.3 Determination of possible binding sites for the cAMP/CRP complex

It is known from the literature that the cAMP molecule is able to form a complex with its receptor protein (CRP). Upon complex formation, this construct can bind to defined recognition sequences upstream of its respective target genes or operons and induce their expression. These sequences are referred to as catabolite responsive elements or CRE sites. By using an already published consensus sequence for CRE sites (Kant *et al.*, 2009) as a motif, the sequence upstream of the sodorifen cluster was screened for possible binding sites of the cAMP/CRP complex. The motif search was performed with the help of the Regulatory Sequence Analysis Tool for prokaryotes (Medina-Rivera *et al.*, 2015) allowing up to 3 mismatches from the consensus

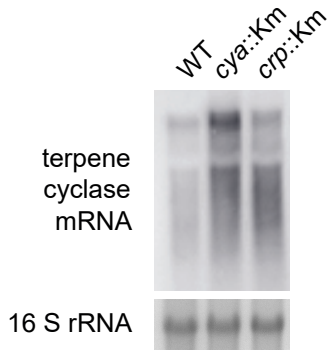

Figure 3.18: Expression of the terpene cyclase in *S. ply.* 4Rx13 *cya*::Km and *crp*::Km in comparison to the wild type. Northern blot of *S. ply.* 4Rx13 wild type, *cya*::Km and *crp*::Km after 24 h cultivation in complex medium (LB). Detection of the terpene cyclase mRNA (upper panel) was conducted using hybridization with a DIG-labelled probe followed by fluorescence measurement for 1 min. 16 S rRNA detection was performed on the same blot as a positive control (lower panel).

sequence. The result can be seen in **figure 3.19**. It was possible to identify two separate potential CRE sites in the sodorifen cluster upstream sequence (CRE1/2) with an identity to the consensus sequence of 85.7 % and 78.6 %, respectively. Furthermore, the potential CRE sites identified in *S. ply.* 4Rx13 were searched for in other sodorifen producer and non-producer strains and their sequences aligned (**figure 3.20**). The results clearly showed that the sodorifen producer strains *S. ply.* 3Re4-18, HRO-C48, and S13 possess potential CRE sites which are 100 % identical to 4Rx13. The only exception is the sodorifen emitting isolate V4 where the CRE1 sequence differs in two positions from the one in 4Rx13, whereas CRE2 is again identical. However, more sequence variations can be found when comparing the sodorifen producing isolate 4Rx13 with the non-producer AS9. In the case of CRE1 the identity amounts to 78.6 % whereas CRE2 was found to be completely missing in *S. ply.* AS9.

3.2.3.1 Influence of the catabolite responsive elements on the sodorifen emission

The effect of the identified cAMP/CRP binding sequences, CRE1 and CRE2, on the sodorifen emission was investigated by constructing deletion mutants where the respective 14 bp sequences were removed and replaced by a kanamycin resistance. For this purpose, again, the *E. coli* Gene Deletion Kit was used (Gene Bridges, Heidelberg, DE; see 2.3.10) to insert a functional cassette by homologous recombination in the sodorifen cluster upstream sequence which, at the same time, causes a deletion of the CRE sequences from the genome of *S. ply.* 4Rx13. As can be seen

```
4Rx13          GGTATTTCCTCTTACTGGCTCGCTATAGATCCATCCGATTTGACACCATGACGCAGGCG 60
CRE            ------------------------------------------------------------

4Rx13          GGAACAAGAAGTAAATGTTATGTTAACTGCAGCGACGCACAATAACCCCACTCTGCAATC 120
CRE            ------------------------------------------------------------

4Rx13          AACATGACCGAGACGCTTCATTACGGTCGGGACAGAGGGTATCGGTGAGCACGTAATATC 180
CRE            ------------------------------------------------------------

                                          CRE1
4Rx13          ATTGGGCTAGCCGACCCCTTTGTTTCTGTAGCCGAAAACATCCATCGCCTGGCTTTAACA 240
CRE            -------------------------TGWAANCGNTNWCA--------------------
                                         **** **** ****

4Rx13          AAAAATAGTTGCACCCTAGCAAAACTCCCACATCGTTGAATTAACATTAAGTTAAACCAT 300
CRE            ------------------------------------------------------------

                                                    CRE2
4Rx13          AAGTTAAAAATATATCACTCGCTGATACTTCATCATAAAATAGTCATCATCATGATTAAT 360
CRE            ----------------------------------TGWAANCGNTNWCA-----------
                                                  * **** ** ****

4Rx13          TTATTTTTCTTAAAAACCGTCATTAAATAAAATTTGACAATGAAAGAAATTCGTCTTTAA 420
CRE            ------------------------------------------------------------

4Rx13          TTAAATT                                                      427
CRE            -------
```

Figure 3.19: Identification of potential CRE sites in the upstream sodorifen cluster sequence in *S. ply.* 4Rx13. Motif search was performed using the Regulatory Sequence Analysis Tool (Medina-Rivera *et al.*, 2015). Line '4Rx13' represents the upstream sodorifen cluster sequence from *S. ply.* 4Rx13 and line 'CRE' the consensus sequence searched for (Kant *et al.*, 2009). A=adenine, T=thymine, C=cytosine, G=guanine, W=A/T, N=any base.

S. ply.	CRE1
4Rx13	TGTAGCCGAAAACA
3Re4-18	TGTAGCCGAAAACA
HRO-C48	TGTAGCCGAAAACA
S13	TGTAGCCGAAAACA
V4	TGTAGCCAAAAATA
AS9	TGTAGCTAAAATCA
	******--***--*

S. ply.	CRE2
4Rx13	TAAAATAGTCATCA
3Re4-18	TAAAATAGTCATCA
HRO-C48	TAAAATAGTCATCA
S13	TAAAATAGTCATCA
V4	TAAAATAGTCATCA
AS9	--------------

Figure 3.20: Alignment of the two potential CRE binding sites in the sodorifen cluster upstream sequence from *S. ply.* 4Rx13. Alignment was performed using the Clustal Omega online software (McWilliam *et al.*, 2013). Strains *S. ply.* 3Re4-18, HRO-C48, S13 and V4 belong to the group of sodorifen producers, whereas *S. ply.* AS9 does not emit sodorifen. Black letters represent nucleotides matching the sequence of *S. ply.* 4Rx13. Red letters indicate differences to the sequence of *S. ply.* 4Rx13. Asterisks mark identities between all strains tested, dashes represent alterations.

in **figures 3.22 A** and **3.21 A**, the deletion of both CRE binding sites upstream of the sodorifen cluster was successful. Using primers specific for the sodorifen cluster upstream region as well as for the resistance cassette and combinations of both, the correct insertion of the functional cassette was unequivocally proven by gaining amplification products of the expected sizes (for details see figure description). Furthermore, the mutations were stably maintained throughout growth in both CRE deletion strains (**figures 3.22 and 3.21 C**). Growth analyses in complex medium (LB) showed no differences between the wild type and its respective CRE negative mutants (**figures 3.22, 3.21 B**). Since the sodorifen emission was demonstrably highest in minimal medium containing 55 mM succinate and the CRE deletion strains were both capable of growing in that medium, the analyses of the volatile spectra were performed in succinate MM. For CRE2::Km it became apparent that no differences in sodorifen emission occurred in comparison to the wild type strain of *S. ply.* 4Rx13 (**figure 3.21 D**). Contrary to this, the CRE1 deletion strain showed a strong reduction in the emission of sodorifen and all of its potential derivatives (**figure 3.22 D**). Apart from this, no qualitative variations were detectable in the VOC profile. Quantitative analysis of the sodorifen emission of CRE1::Km in comparison to the *S. ply.* 4Rx13 WT were performed in minimal medium supplemented with succinate by relating the amount of emitted sodorifen to the number of viable cells (**figure 3.23**). In the wild type, the sodorifen emission increased fourfold from 100 % to 441 % after 48 h cultivation and decreased afterwards again to 207 %. For CRE1::Km a steady upward trend was observable and in contrast to the WT, the measured sodorifen amounts only reached values ranging from 12.5 % to 48.6 % representing a reduction in sodorifen emission of 76 %-92 %.

Furthermore, the influence of CRE1 deletion on the sodorifen cluster expression was assayed. Exemplarily, the mRNA level of the terpene cyclase was investigated by Northern blot after 24 h cultivation of both, the wild type and the CRE1 mutant, in minimal medium supplemented with succinate (**figure 3.24**). For this purpose, a DIG-labelled probe specifically binding the terpene cyclase mRNA was generated and hybridized to it. Detection of bound probes using fluorescence measurement revealed a significantly higher level of terpene cyclase mRNA in the wild type of *S. ply.* 4Rx13 in comparison to the CRE1 deletion strain. For quantification, the signal intensity of the wild type was used as reference (100 %) resulting in an expression level of 21.14 % for the terpene cyclase in CRE1::Km. The 16 S rRNA was used as a positive control and ensured equal RNA load on the membrane previous to hybridization. In conclusion, deletion of the potential cAMP/CRP binding site CRE2 from the sodorifen cluster upstream sequence caused no effect concerning the sodorifen emission, whereas deletion of CRE1 lead to a significant decrease of up to

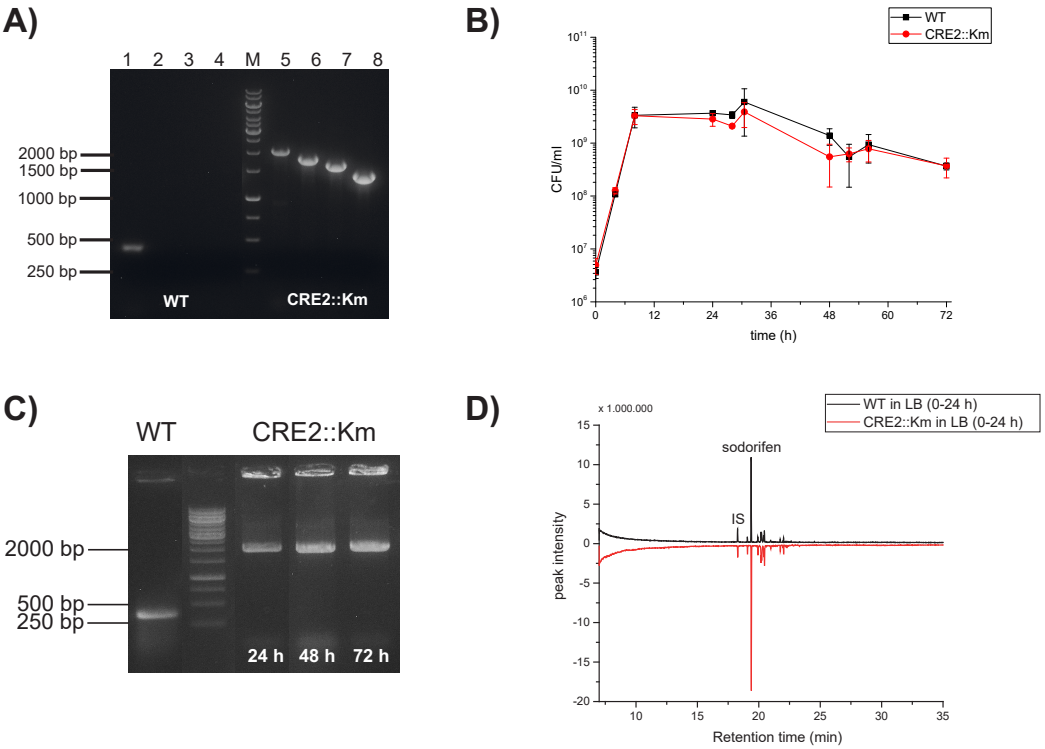

Figure 3.21: Characterization of the CRE2::Km deletion mutant of *S. ply.* 4Rx13. A) Verification of correct insertion of the functional cassette by PCR. In lanes 1-4 results for the *S. ply.* 4Rx13 wildtype are shown and in lanes 5-8 for the CRE2 mutant. Lanes 1 & 5 represent amplification products with primers for the sodorifen cluster upstream region (434 bp for the WT; 2.06 kb for CRE2::Km). Lanes 4 & 8 depict amplification products with primers specific for the functional cassette (1.4 kb). Lanes 2 & 6 show PCR products after amplification with primers specific for the upstream region and the functional cassette (left border; 1.82 kb),as well as lanes 3 & 7 (right border; 1.66 kb). M = marker (GeneRuler® 1 kb, Thermo Fisher Scientific, Madison, USA). **B)** Growth comparison of *S. ply.* 4Rx13 WT and CRE2::Km in complex medium (LB). The living cell number (CFU/ml) was determined periodically for up to 72 h. Error bars depict standard deviation (n=3). **C)** Stability of the mutation of CRE2::Km in *S. ply.* 4Rx13 was determined in 24 h intervals for up to 72 h of cultivation in complex medium (LB) using PCR. **D)** Volatile spectrum of *S. ply.* 4Rx13 wildtype (black) in comparison to the CRE2 deletion mutant (red) during cultivation in minimal medium +55 mM succinate (0-24 h). Volatiles were sampled using the VOC collection system and analyzed by GC/MS. IS = internal standard (nonylacetate, 5 ng).

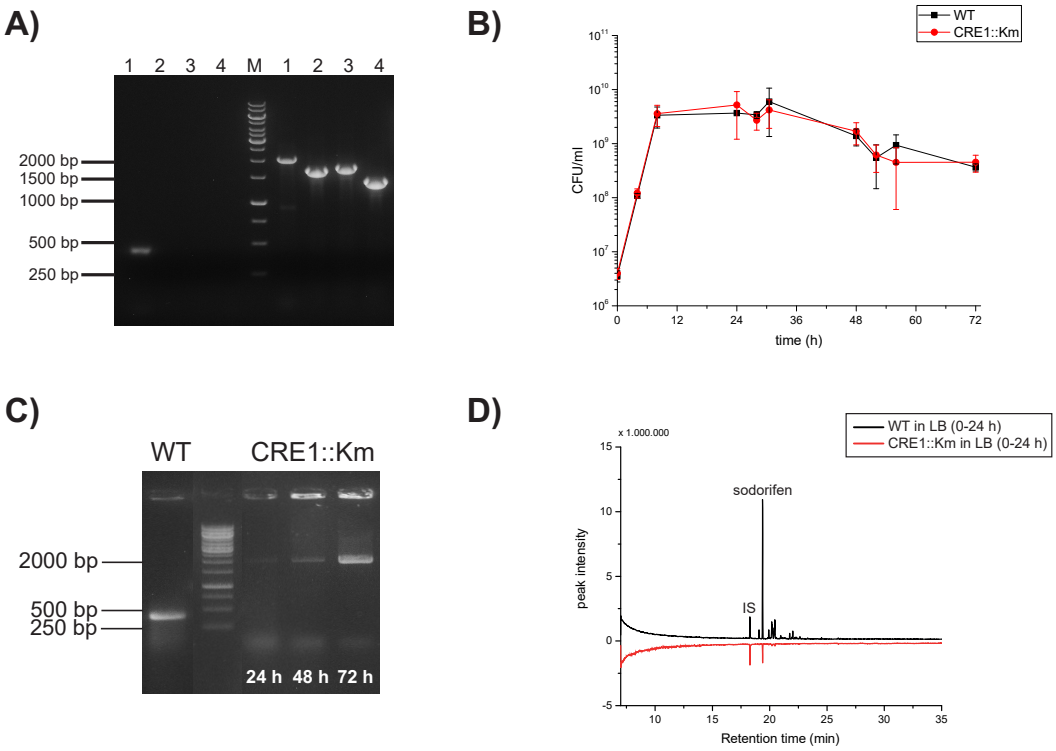

Figure 3.22: Characterization of the CRE1::Km deletion mutant of *S. ply.* 4Rx13. A) Verification of correct insertion of the functional cassette by PCR. In lanes 1-4 results for the *S. ply.* 4Rx13 wildtype are shown and in lanes 5-8 for the CRE1 mutant. Lanes 1 & 5 represent amplification products with primers for the sodorifen cluster upstream region (434 bp for the WT; 2.06 kb for CRE1::Km). Lanes 4 & 8 depict amplification products with primers specific for the functional cassette (1.4 kb). Lanes 2 & 6 show PCR products after amplification with primers specific for the upstream region and the functional cassette (left border; 1.68 kb),as well as lanes 3 & 7 (right border; 1.79 kb). M = marker (GeneRuler® 1 kb, Thermo Fisher Scientific, Madison, USA). **B)** Growth comparison of *S. ply.* 4Rx13 WT and CRE1::Km in complex medium (LB). The living cell number (CFU/ml) was determined periodically for up to 72 h. Error bars depict standard deviation (n=3). **C)** Stability of the mutation of CRE1::Km in *S. ply.* 4Rx13 was determined in 24 h intervals for up to 72 h of cultivation in complex medium (LB) using PCR. **D)** Volatile spectrum of *S. ply.* 4Rx13 wildtype (black) in comparison to the CRE1 deletion mutant (red) during cultivation in minimal medium + 55 mM succinate (0-24 h). Volatiles were sampled using the VOC collection system and analyzed by GC/MS. IS = internal standard (nonylacetate, 5 ng).

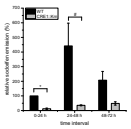

Figure 3.23: Relative sodorifen emission of CRE1::Km in minimal medium + succinate. *S. ply.* 4Rx13 wild type (black bars) and CRE1::Km mutant (grey bars) were cultivated for 72 h in minimal medium + 55 mM succinate. Volatiles were collected using the VOC collection system in 24 h time intervals. The relative sodorifen emission was calculated in correlation to the living cell number. The sodorifen emission of the wild type from 0-24 h was defined as 100 %. Error bars indicate standard deviations (n=3).

92 % in sodorifen emission (figures 3.22 & 3.23). Moreover, also the expression level of the terpene cyclase gene was reduced in the CRE1 deletion strain by about 79 % in comparison to the wild type of *S. ply.* 4Rx13 (figure 3.24).

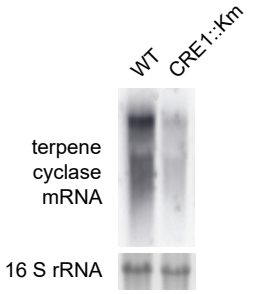

Figure 3.24: Expression of the terpene cyclase in *S. ply.* 4Rx13 wild type and CRE1::Km. Northern blot was performed using RNA from *S. ply.* 4Rx13 wild type (WT) and CRE1::Km mutant strain after 24 h cultivation in minimal medium supplemented with 55 mM succinate. Terpene cyclase mRNA was hybridized with a DIG-labelled probe (upper panel) and subsequently detected using fluorescence measurement for 30 s. 16 S rRNA was detected as a positive control on the same blot (lower panel).

3.3 Regulation of sodorifen emission by quorum sensing

Since the sodorifen emission was found to begin during transition from the exponential growth phase to the stationary phase, i.e. at high cell densities, the role of quorum sensing (QS) mechanisms in sodorifen biosynthesis was assessed. As a first approach, genes responsible for the production and reception of QS signals were searched in the genome of *S. ply.* 4Rx13. Secondly, insertional deletion mutagenesis was performed on the identified genes and the volatile profiles of the generated mutants were analyzed with special regards to the emission of sodorifen.

3.3.1 Genome mining for *lux*I/R homologues in *Serratia* spp.

In *Escherichia coli* strains, the proteins for production and perception of the quorum sensing signals are encoded by the genes *lux*I (QS signal synthase) and *lux*R (QS signal receptor). Previous analyses concerning the QS systems in *Serratia* strains revealed homologous genes of *lux*I/R in the isolate *S. ply.* G3 and interestingly also in the sodorifen producer strain *S. ply.* HRO-C48, referred to as *spl*I/R (van Houdt *et al.*, 2007; Liu *et al.*, 2007). Using *spl*I/R as a template, BLAST analysis was performed on the genome of *S. ply.* 4Rx13 for identification of homologues (NCBI Resource Coordinators, 2013). As a result, two genes were found, annotated as *esa*I/R, matching the query sequence to 99 %. According to the genomic location, convergent transcription with an overlap of 28 bp between both genes was found (**figure 3.25**). Furthermore, other *Serratia* isolates were screened for orthologous genes of *esa*I/R (**table 3.1**). It became apparent that all sodorifen producer strains possess quorum sensing genes with 99 % identity to the 4Rx13 system. The only exception was *S. ply.* V4 where only an orthologue for *esa*R was found but not for *esa*I. Also, the identity of the QS receptor to 4Rx13 only amounted up to 83 %. Additional to the sodorifen producer strains *S. ply.* HRO-C48, 3Re4-18, and S13, also the isolates RVH1 and G3 showed quorum sensing related genes with very high identity to *S. ply.* 4Rx13 (99 %).

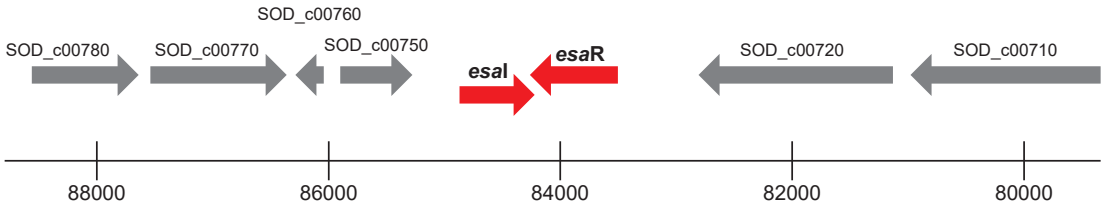

Figure 3.25: Genomic position of *esa*I/R genes in *S. ply.* 4Rx13. The scale indicates the location of *esa*I/R in the genome of *S. ply.* 4Rx13.

Table 3.1: Nucleotide sequence comparison of *esa*l/R from *S. ply.* 4Rx13 with other *Serratia* spp. Identities were determined using the Nucleotide BLAST tool (NCBI Resource Coordinators, 2013). For isolates marked with 'n.a.' no information is available concerning their sodorifen emitting properties. + = sodorifen-producer; – = sodorifen non-producer

Serratia strain	sodorifen producer	*esa*l		*esa*R	
		coverage (%)	identitiy (%)	coverage (%)	identity (%)
S. plymuthica HRO-C48	+	100	99	100	99
S. ply. 3Re4-18	+	100	99	100	99
S. ply. 3Rp8	n.a.	100	99	100	99
S. ply. S13	+	100	99	100	99
S. ply. RVH1	n.a.	100	99	100	99
S. ply. G3	n.a.	100	99	100	99
S. ply. AS9	–	0	0	98	84
S. ply. AS12	–	0	0	98	84
S. ply. AS13	n.a.	0	0	98	84
S. ply. PRI-2C	–	0	0	100	84
S. ply. V4	+	0	0	99	83
S. proteamaculans 91	n.a.	100	83	100	86
S. pr. 568	–	0	0	45	84
S. liquefaciens FDAARGOS_125	n.a.	99	80	99	87
S. l. HUMV-21	n.a.	99	80	99	86
S. marcescens H30	n.a.	98	75	99	83
S. m. AS-1	n.a.	98	75	99	83
S. m. DB11	–	0	0	0	0
S. odorifera DSM4582	–	0	0	0	0

Interestingly, all, so far tested, sodorifen non-producers (*S. pr.* 568, *S. m.* DB11, *S. o.* DSM4582 and *S. ply.* PRI-2C and AS9) were, in each case, missing either one or both of the QS genes from *S. ply.* 4Rx13.

3.3.2 Role of *esa*l/R in the regulation of sodorifen emission

In previous works, transcriptome analyses were performed with the sodorifen producer *S. ply.* 4Rx13 in comparison to its close relative and non-producing strain AS9 under sodorifen producing conditions (i.e. growth in minimal medium with succinate; Domik *et al.* (2016b)). The transcription level of each expressed gene is given in NPKM values (nucleotide activity per kilobase of exon model per million mapped reads) representing the amount of sequenced nucleotides per gene. For the two identified quorum sensing related genes, *esa*l/R, an expression was solely observable in the sodorifen producer *S. ply.* 4Rx13, whereas it was completely ab-

sent in the non-producing strain AS9 (**figure 3.26**). Interestingly, the expression of *esa*R was increased fourfold in comparison to *esa*I and other adjacent genes in *S. ply.* 4Rx13. At both time points investigated by RNA sequencing (24 h and 48 h), the expression levels of *esa*I & *esa*R remained almost constant (*esa*I 24 h = 25, 48 h = 31; *esa*R 24 h = 117, 48 h = 131; Domik *et al.*, unpublished).

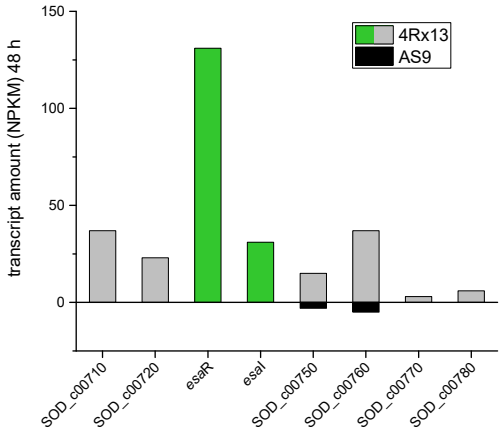

Figure 3.26: Expression of *esa*I/R in *S. ply.* 4Rx13 and AS9. Expression levels are represented as NPKM values (nucleotide activities per kilobase of exon model per million mapped reads) after 48 h cultivation in minimal medium supplemented with 55 mM succinate. Green bars indicate transcription levels of *esa*I/R. Grey bars show expression of surrounding genes in *S. ply.* 4Rx13, black bars in AS9.

Knockout of *esa*I/R in *S. ply.* 4Rx13 allowed for a closer investigation of the influence of quorum sensing mechanisms on the sodorifen biosynthesis and/or emission. Characterizations of the obtained insertion mutants *esa*I::Km and *esa*R::Km are depicted in **figures 3.27**/**3.28**. Using PCR the correct insertion of the functional cassette was verified for both mutants. For this purpose, primers specific for the corresponding gene, the inserted cassette and combinations of both were used. As a control the wild type was analyzed using the same primer combinations. As a result, amplification products with expected sizes were gained (**A**). Furthermore, amplification with gene-specific primers lead to an amplification product with an increased size of ca. 1.6 kb in the insertion mutants in comparison to the wild type. Also, amplification products with insert-specific primers were only obtained in the mutant strains and not in the wild type, proving presence of the functional cassette only in *esa*I/R::Km. PCR analysis, using gene-specific primers, was also used to evaluate the stability of the mutation for up to 72 h of cultivation in minimal medium containing succinate as the sole carbon source (**C**). Left of the marker, the amplification product of the wild type gene is depicted with an expected size of 564 bp for *esa*I and 697 bp for *esa*R. On the right side, the PCR products for the insertional deletion mutants *esa*I::Km (2.2 kb) and *esa*R::Km (2.3 kb) after 24, 48 and 72 h of growth are shown, proving a stable insertion of the functional cassette in the two

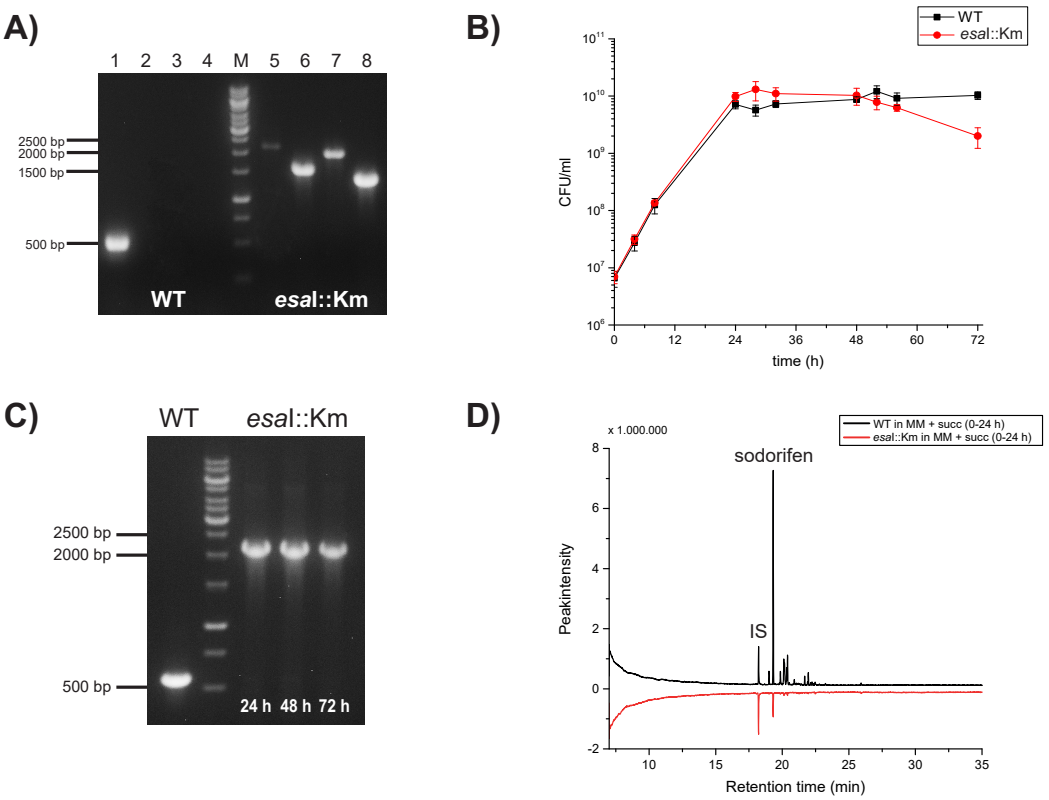

Figure 3.27: Characterization of the *esa*l::Km deletion mutant of *S. ply.* 4Rx13. A) Verification of correct insertion of the functional cassette by PCR. In lanes 1-4 results for the *S. ply.* 4Rx13 wildtype are shown and in lanes 5-8 for the *esa*l mutant. Lanes 1 & 5 represent amplification products with gene-specific primers for the *esa*l gene (564 bp for the WT; 2.19 kb for *esa*l::Km). Lanes 4 & 8 depict amplification products with primers specific for the functional cassette (1.4 kb). Lanes 2 & 6 show PCR products after amplification with gene- and cassette-specific primers (1.6 kb; left border),as well as lanes 3 & 7 (right border; 2.0 kb). M = marker (GeneRuler® 1 kb, Thermo Fisher Scientific, Madison, USA). **B)** Growth comparison of *S. ply.* 4Rx13 WT and *esa*l::Km in minimal medium + 55 mM succinate. The living cell number (CFU/ml) was determined periodically for up to 72 h. Error bars depict standard deviation (n=3). **C)** Stability of the mutation of *esa*l in *S. ply.* 4Rx13 was determined in 24 h intervals for up to 72 h of cultivation in minimal medium + 55 mM succinate using PCR. **D)** Volatile spectrum of *S. ply.* 4Rx13 wildtype (black) in comparison to the *esa*l mutant (red) during cultivation in complex medium (LB; 0-24 h). Volatiles were sampled using the VOC collection system and analyzed by GC/MS. IS = internal standard (nonylacetate, 5 ng).

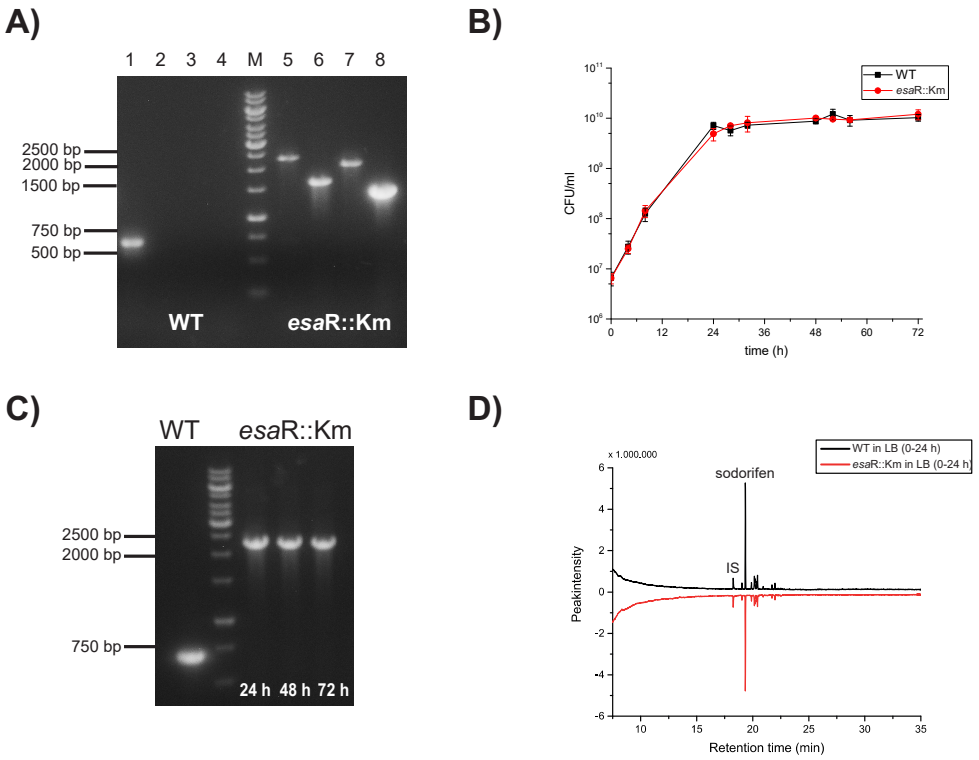

Figure 3.28: Characterization of the *esa*R::Km deletion mutant of *S. ply.* 4Rx13. A) Verification of correct insertion of the functional cassette by PCR. In lanes 1-4 results for the *S. ply.* 4Rx13 wildtype are shown and in lanes 5-8 for the *esa*R mutant. Lanes 1 & 5 represent amplification products with gene-specific primers for the *esa*R gene (697 bp for the WT; 2.32 kb for *esa*R::Km). Lanes 4 & 8 depict amplification products with primers specific for the functional cassette (1.4 kb). Lanes 2 & 6 show PCR products after amplification with gene- and cassette-specific primers (1.64 kb; left border),as well as lanes 3 & 7 (right border; 2.10 kb). M = marker (GeneRuler® 1 kb, Thermo Fisher Scientific, Madison, USA). **B)** Growth comparison of *S. ply.* 4Rx13 WT and *esa*R::Km in minimal medium + 55 mM succinate. The living cell number (CFU/ml) was determined periodically for up to 72 h. Error bars depict standard deviation (n=3). **C)** Stability of the mutation of *esa*R in *S. ply.* 4Rx13 was determined in 24 h intervals for up to 72 h of cultivation in minimal medium + 55 mM succinate using PCR. **D)** Volatile spectrum of *S. ply.* 4Rx13 wildtype (black) in comparison to the *esa*R mutant (red) during cultivation in complex medium (LB; 0-24 h). Volatiles were sampled using the VOC collection system and analyzed by GC/MS. IS = internal standard (nonylacetate, 5 ng).

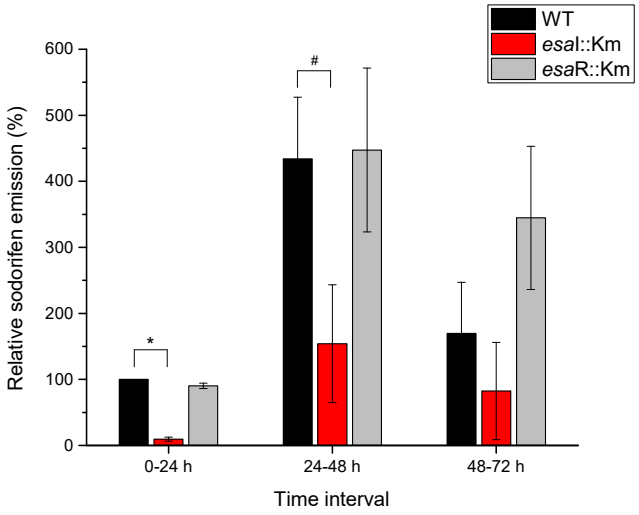

Figure 3.29: Relative sodorifen emission of *esa*I::Km and *esa*R::Km in minimal medium + succinate. *S. ply.* 4Rx13 wild type (black bars), *esa*I::Km (grey bars) and *esa*R::Km (red bars) were cultivated for 72 h in minimal medium + 55 mM succinate. Volatiles were collected using the VOC collection system in 24 h time intervals. The relative sodorifen emission was calculated in correlation to the living cell number. The sodorifen emission of the wild type from 0-24 h was defined as 100 %. Error bars indicate standard deviations (n=3). * $p \leq 0.001$; # $p < 0.05$.

quorum sensing genes in *S. ply.* 4Rx13. As a third step, growth of the mutant strains was investigated in comparison to the wild type. This was also performed in minimal medium supplemented with succinate by documenting the living cell number at distinct time points from 0-72 h (**B**). It became apparent that insertional deletion of the two quorum sensing related genes *esa*IR caused no growth alteration in *S. ply.* 4Rx13. Finally, the volatile spectra of the mutant strains were analyzed in comparison to the wild type of *S. ply.* 4Rx13 (**D**). For this purpose, the wild type of *S. ply.* 4Rx13 and the mutant strains *esa*I/R::Km were cultivated in VOC collection systems in succinate minimal medium for up to 72 h. In 24 h time intervals the volatiles were trapped on the adsorbent Porapak^{TM}, eluted with a solvent and analyzed using GC-MS. In both mutant strains no qualitative variations could be observed in comparison to the wild type. Also, for *esa*R::Km there were no quantitative differences in the emission of sodorifen observable. In contrast to this, sodorifen emission was appreciably reduced in *esa*I::Km in comparison to the wild type. Quantitative evaluation of the sodorifen emission was performed, considering the living cell number (CFU/ml) and related to the wild type emission from 0-24 h (100 %; **figure 3.29**). It became apparent that the sodorifen emission in the wild type strain is increasing until 24-48 h from 100 % to 434 % and slightly decreasing until 72 h of cultivation in minimal medium supplemented with 55 mM succinate to 169 %. Overall, no significant differences in the relative sodorifen emission were detected for *esa*R::Km.

Only during the last time interval tested (48-72 h), the mutant strain defective in the quorum sensing signal receptor showed an increased emission of 344 % in comparison to the wild type. However, for *esa*I::Km a significant inhibition of the sodorifen emission was observed ranging from 51 % (48-72 h) to 90 % (0-24 h). This leads to the conclusion that defects in the quorum sensing signal receptor (*esa*R::Km) have no effects on the sodorifen biosynthesis. In contrast to this, malfunction of the signal production (*esa*I::Km) results in strong reduction of sodorifen emission in *S. ply.* 4Rx13.

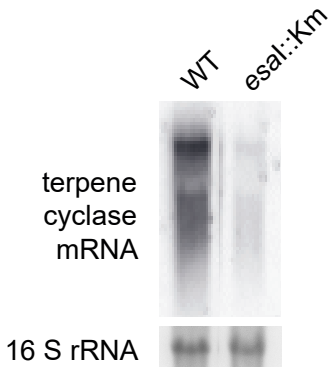

Figure 3.30: Expression of the terpene cyclase in *S. ply.* 4Rx13 wild type and *esa*I/R::Km. Northern blot was performed using RNA from *S. ply.* 4Rx13 wild type (WT) and *esa*I::Km mutant strain after 24 h cultivation in minimal medium supplemented with 55 mM succinate. Terpene cyclase mRNA was hybridized with a DIG-labelled probe (upper panel) and subsequently detected using fluorescence measurement for 30 s. 16 S rRNA was detected as a positive control (lower panel) in the same way.

In an attempt to identify the reason for the reduced sodorifen emission in *esa*I::Km, expression analysis of the sodorifen cluster was performed by Northern blot (**figure 3.30**). For this purpose, total RNA was isolated from *S. ply.* 4Rx13 WT and *esa*I::Km after 24 h cultivation in minimal medium with succinate. After blotting on a nylon membrane, specifically the terpene cyclase mRNA was targeted using a DIG-labelled probe. After visualization by fluorescence measurement, it became apparent that the expression level of the terpene cyclase gene is considerably higher in the wild type strain in comparison to the *esa*I mutant. Software-based quantification revealed an inhibition of sodorifen cluster expression in *esa*I::Km of 93 % in relation to the wild type, perfectly reflecting the reduced sodorifen emission of this strain.

3.3.3 Identification of a potential second QS system

Since inactivation of the enzyme responsible for synthesis of the quorum sensing signal receptor (*esa*R) in *S. ply.* 4Rx13 exhibited the same phenotype like the wild

type, the question arose if an additional QS system is present in 4Rx13 which could complement the dysfunction of *esa*R. According to the literature, bacteria often possess a quorum sensing system for interspecies cross-talk, characterized by the enzyme S-ribosylhomocysteine lyase which is highly conserved among different bacterial species (Federle and Bassler, 2003; Vendeville *et al.*, 2005). This enzyme is encoded by the *lux*S gene and leads to production of the quorum sensing signal termed autoinducer-2 (AI-2). Taking the nucleotide sequence from *lux*S in *E. coli* K-12 MG1655 as a reference, BLAST analysis was performed using discontiguous megablast. The results showed an orthologous *lux*S gene in *S. ply.* 4Rx13 with a nucleotide identity of 79 % to the query sequence. By comparing the amino acid sequences of the corresponding enzymes, the identity of the *S. ply.* 4Rx13 protein was 85 % to *E. coli*. To assess a potential role of *lux*S in the biosynthesis and emission of sodorifen, insertional deletion mutation on that particular gene was performed (Teichmann, 2016). Further analyses of this mutant revealed that the phenotype was not differing from the wild type of *S. ply.* 4Rx13 concerning growth behaviour and volatile profile (Teichmann, 2016). Most importantly, after quantification of the sodorifen emission of *lux*S::Km in relation to the living cell number and referring to the wild type emission after 24 h of cultivation in complex medium (100 %), no significant differences were observable (**figure 3.31**). Only during the time interval from 24-48 h the relative sodorifen emission was lower in the *lux*S::Km mutant (134 %) than in the wild type (220 %). In conclusion, it appears that the interspecies quorum sensing signal AI-2 encoded by the *lux*S gene plays no crucial role in sodorifen biosynthesis.

3.3.4 The effect of cell-free supernatants on the sodorifen emission in *Serratia* spp.

Previous experiments revealed an influence of quorum sensing mechanisms on the sodorifen emission. Furthermore, it was shown that the sodorifen non-producing isolate *S. ply.* AS9 only possesses an orthologue for one of the two quorum sensing genes in *S. ply.* 4Rx13, *esa*R. *Esa*R codes for the quorum sensing signal receptor, whereas *esa*l represents the signal synthase gene. Therefore, it was assumed that a missing QS signal in *S. ply.* AS9 could contribute to the sodorifen negative phenotype of this particular strain. Since quorum sensing signal molecules are excreted into the surrounding medium, cell-free culture supernatants were collected from the sodorifen producer *S. ply.* 4Rx13 and applied on cultures of the non-producing strain AS9. For this purpose, 70 ml of fresh complex medium was mixed with 30 ml of the extracted culture-supernatant and subsequently inoculated with *S. ply.* AS9. Following this, the volatile emission of AS9 was analyzed using the VOC collection

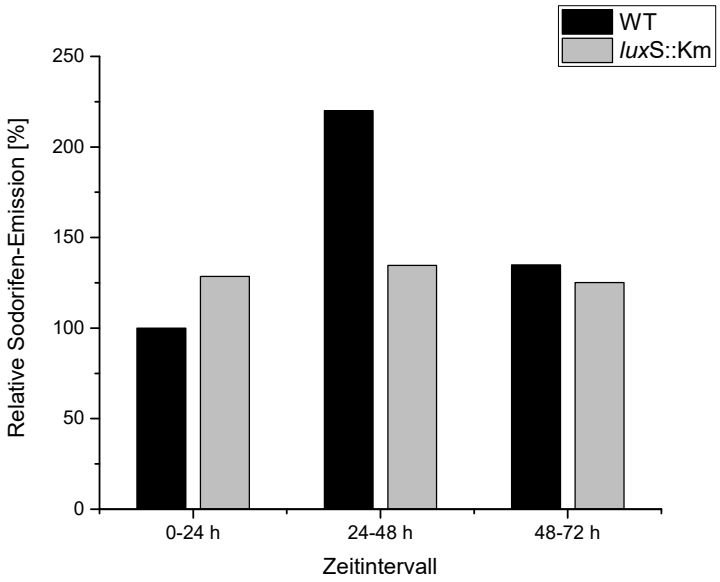

Figure 3.31: Relative sodorifen emission of *lux*S::Km in complex medium. *S. ply.* 4Rx13 wild type (black bars) and *lux*S::Km (grey bars) were cultivated for 72 h in complex medium (LB). Volatiles were collected using the VOC collection system in 24 h time intervals. The relative sodorifen emission was calculated in correlation to the living cell number. The sodorifen emission of the wild type from 0-24 h was defined as 100 %. n=1

system and coupled GC/MS (**figure 3.32** red line). As a control, AS9 was cultivated in complex medium without addition of the supernatant (black line). Qualitatively, no differences in the volatile spectra were detectable. Only concerning the quantity, variations became apparent. Whereas compounds 1-9 were emitted in higher amounts in the AS9 control culture, compounds 10-12 were more abundant in cultures containing the 4Rx13 supernatant. But most importantly, no emission of sodorifen was detectable upon addition of the supernatant from *S. ply.* 4Rx13 leading to the conclusion that artificially added quorum sensing molecules are not sufficient to induce the biosynthesis of sodorifen in the non-producing strain AS9.

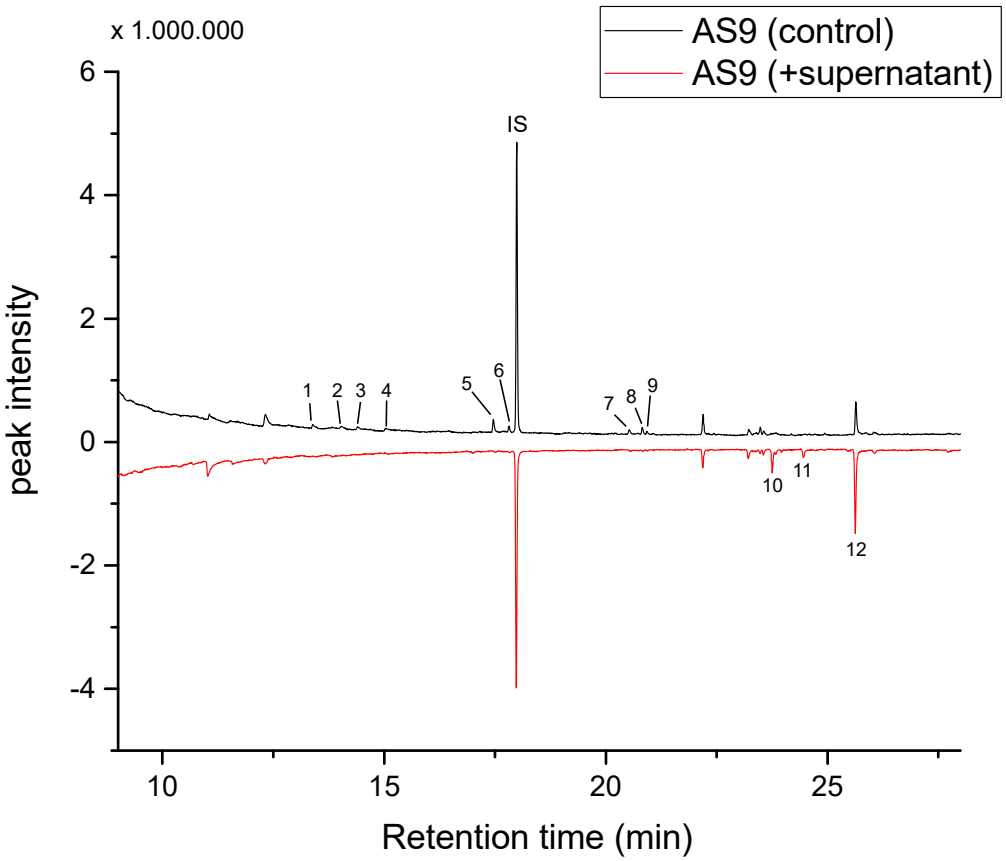

Figure 3.32: Volatile emission profile of *S. ply.* AS9 in complex medium compared to complex medium supplemented with cell-free supernatants from *S. ply.* 4Rx13. The volatiles were sampled using the VOC collection system in 24 h time intervals and analyzed by GC/MS. IS = internal standard (nonylacetate, 5 ng).

4 Discussion

The unique volatile organic compound sodorifen was firstly discovered in 2005 (Kai, 2005). Since then, the aim of research efforts was to assign a biological function to this unusual molecule. To achieve this goal, several attempts have been made, including genome analyses of sodorifen-producing (*S. ply.* 4Rx13, HRO-C48, 3Re4-18, & S13) versus non-producing isolates (e.g. *S. ply.* AS9), proteome and also transcriptome analyses (Weise, 2013; Domik, 2012; Domik *et al.*, 2016b). Using only genome- and proteome-based data, a list of few potential sodorifen biosynthesis genes was established which all turned out to be not essential for this process. In contrast, transcriptome comparison of *S. ply.* 4Rx13 with its genetically close, but sodorifen-non producing relative AS9 revealed more than 300 genes being expressed only in the sodorifen producer 4Rx13 (Domik *et al.*, unpublished). Among them, a four-gene cluster was identified, annotated as SOD_c20750-SOD_c20780, which encodes for a terpene cyclase, a methyltransferase, a DOXP synthase and an isopentenyl pyrophosphate isomerase, respectively (Domik *et al.*, 2016a). Moreover, inactivation of three out of these four genes lead to a sodorifen-negative phenotype providing evidence for the crucial role of this cluster for the sodorifen biosynthesis, which is why it is also referred to as the "sodorifen cluster" (Domik *et al.*, 2016a).

Having this set of four genes now in hand, it was possible to assess conditions which favour cluster expression and thus, sodorifen production. Therefore, the aim of this thesis was to shed light on the regulatory mechanisms behind the biosynthesis of the unique volatile organic compound sodorifen to be ultimately able to draw conclusions concerning the function of sodorifen in nature. For this purpose, expression analyses of the sodorifen cluster in different producer strains and under different cultivation conditions were performed and the effect of several transcriptional regulators on the sodorifen production was assessed using functional knockout by insertional deletion mutagenesis.

4.1 Regulation of the sodorifen emission in different *Serratia* spp.

4.1.1 Differential regulation in varying producer strains

The phenomenon of sodorifen emission is not widely distributed among bacteria. So far, only five sodorifen producing bacterial isolates could be identified all belonging to the species *Serratia plymuthica* (Weise *et al.*, 2014; Domik *et al.*, 2016b). Previ-

ously, it has been found that among these five strains, *S. ply.* 4Rx13 emits the highest amounts of sodorifen (Domik *et al.*, 2016b). The present study went one step further by not only comparing the absolute sodorifen amounts emitted by the different producer strains, but also by determining the relative sodorifen emission per living cell. Furthermore, the sodorifen producing isolate *S. ply.* S13 (Müller *et al.*, 2013) was included for the first time.

As a result, *S. ply.* 4Rx13 was found to emit significantly more sodorifen, reaching levels which were up to 800 times higher than by the other producer strains investigated. The isolates *S. ply.* HRO-C48 and 3Re4-18 emitted almost the same amounts of sodorifen, followed by S13 and V4. This indicates that *S. ply.* 4Rx13 does not only emit the highest sodorifen amount, but also that the other producers differ considerably in their emission levels (6.5 - 0.1 %). One possible explanation for this disparity could be a diverging expression of the sodorifen biosynthesis genes. In a first attempt it was proven by RT-PCR that all four genes of the sodorifen biosynthesis cluster are transcribed in one polycistronic mRNA in *S. ply.* 4Rx13 (Domik *et al.*, 2016a). Moreover, it was demonstrated in this work that expression of the sodorifen cluster genes was detectable in all sodorifen producing isolates. Following this, Northern blot technique used in this study allowed for detection of the sodorifen cluster mRNA using a probe specific for the terpene cyclase of *S. ply.* 4Rx13. The expression levels of the sodorifen cluster were differing considerably among sodorifen producing isolates. As expected, sodorifen cluster expression was reduced in *S. ply.* S13, V4 and HRO-C48 in comparison to the control (*S. ply.* 4Rx13) by 5-50 fold, which correlates with the reduced sodorifen emission in these strains. To exclude less efficient binding of the terpene cyclase probe in sodorifen producers other than 4Rx13, the nucleotide sequences of the terpene cyclase genes of all producer strains were compared which revealed only 14-15 nucleotides differences in *S. ply.* HRO-C48, 3Re4-18, S13 and V4 in comparison to *S. ply.* 4Rx13 (results not shown). Therefore, it was concluded that the probe was capable to bind properly to the sodorifen cluster mRNA resulting in accurate reflection of the expression status of the sodorifen cluster in all strains tested. Consequently, sodorifen cluster expression in the producer strains *S. ply.* HRO-C48, S13 and V4 has to be differentially regulated compared to *S. ply.* 4Rx13.

One possible mechanism would be by transcriptional regulation of the sodorifen cluster expression. To assess this hypothesis, the upstream sequence of the sodorifen cluster was analyzed in all known sodorifen producers as well as in the non-producer *S. ply.* AS9. The results revealed very high homology of the nucleotide sequence directly upstream of the sodorifen cluster among all producer strains (94-98 %), whereas the non-producer *S. ply.* AS9 showed many deviations

to the sequence present in *S. ply.* 4Rx13, including two large deletions. Possibly, these deletions lead to an inability of potential transcription factors to bind to the upstream sequence in *S. ply.* AS9, which in turn results in non-expression of the sodorifen cluster genes and therefore in the sodorifen-negative phenotype of this isolate. Consistently, Mahmood and Khan (1990) could show that deletion of only 34 bp from the upstream sequence of the staphylococcal enterotoxin B gene (*seb*) lead to a complete repression of gene expression.

In contrast, the sodorifen-producers *S. ply.* 4Rx13, HRO-C48, 3Re4-18, S13 and V4 all shared a very high identity of their upstream sequences but still the latter four exhibited only low sodorifen production. One explanation can be found at the end of the upstream sequence which is either elongated by eight nucleotides (in *S. ply.* HRO-C48 and S13) or reveals a missing guanine (*S. ply.* 3Re4-18 and V4) right before the start-codon of the first gene in the sodorifen cluster. These deviations could lead to changes in the distance of the potential promoter sequence to the transcriptional starting site and therefore, to a less efficient transcription of the sodorifen cluster genes. To investigate whether a promoter shift is responsible for the lower sodorifen emission values in *S. ply.* HRO-C48, 3Re4-18, S13 and V4, the transcriptional start sites in the respective strains should be determined using 5'-RACE PCR. Another explanation could be that additional transcription factors are involved in induction of the sodorifen cluster expression whose binding to the operator of the sodorifen cluster is impaired in the low-producer strains compared to *S. ply.* 4Rx13, because of the overall differences in the nucleotide upstream sequence. Identification of potential transcription factors acting on the sodorifen cluster expression are subject of current investigations.

Surprisingly, *S. ply.* 3Re4-18 which emitted 15-250 times less sodorifen compared to *S. ply.* 4Rx13, produced higher expression values of the terpene cyclase gene exceeding the one of 4Rx13 by one third. Until now, there is no proven explanation for this contradictory result. Nevertheless, it can be hypothesized that in case of *S. ply.* 3Re4-18 the downregulation of sodorifen emission is not due to a decrease in expression of the biosynthetic genes, but rather because of post-transcriptional regulatory mechanisms. Further indication of non-transcriptional regulation of sodorifen emission has been observed previously when Weise (2013) showed that addition of succinate to minimal medium significantly increased sodorifen emission, whereas upon further addition of methionine no sodorifen was detectable anymore in the headspace of *S. ply.* 4Rx13. In this case, preliminary expression studies under both cultivation conditions (MM + succinate and MM + succinate + methionine) demonstrated that although sodorifen emission was completely abolished by addition of methionine, sodorifen cluster expression was not affected at all (for details

see **supplemental figure S1**). Interestingly, it cannot be methionine itself that leads to complete repression of sodorifen emission since feeding a mixture of alanine, threonine and methionine to *S. ply.* 4Rx13 even lead to sodorifen emissions more than twice as high as in complex medium (Weise, 2013). Furthermore, differential mRNA turn-over or increased half-life of the sodorifen cluster mRNA would be plausible explanations for the observed disparities in *S. ply.* 3Re4-18 in comparison to *S. ply.* 4Rx13.

Summarizing the above, it can be stated that sodorifen biosynthesis differs in assorted species due to either transcriptional or post-transcriptional regulatory processes. The mechanisms underlying the post-transcriptional regulations are still not understood and will be part of further research.

4.1.2 Temporal regulation of sodorifen cluster expression

Apart from the disparate regulation of sodorifen emission, also consistent regulatory mechanisms among the producer strains could be identified in this study, for example, the temporal regulation of sodorifen biosynthesis. In previous studies it was reported that sodorifen emission in *S. ply.* 4Rx13 increased from 24-72 h during cultivation in minimal medium supplemented with succinate (Weise, 2013). Consequently, it was of interest to investigate the expression status of the sodorifen cluster throughout growth. Therefore, total RNA of *S. ply.* 4Rx13 cultivated in succinate containing medium was isolated at different time points (6-72 h) and expression of the terpene cyclase mRNA was detected using Northern blot. Transcriptional activity of the sodorifen cluster became visible only after 12 h of growth in MM + succinate representing the time of transition from the phase of exponential to stationary growth in *S. ply.* 4Rx13. Nevertheless, using the highly sensitive RT-PCR technique, Bernhardt (2015) could show that weak expression of the sodorifen cluster was detectable as early as 5 h post inoculation.

Therefore, it can be hypothesized that the sodorifen biosynthesis genes are poorly expressed during exponential growth where still many nutrients are available. As soon as nutrients become limited, cells stop to divide and enter stationary phase which goes along with induction of sodorifen cluster expression and thus, sodorifen emission. Interestingly, it was found that in all sodorifen producing strains tested, expression of the four-gene cluster was highest after 24 h cultivation in MM + succinate and afterwards decreased drastically until it was no longer detectable in any of the strains after 72 h of cultivation. This is in contrast to previous results of the transcriptome analysis by Domik et al. (unpublished), where the transcriptional activity of the sodorifen cluster was increased after 48 h cultivation in minimal medium + succinate by almost 50 % in comparison to values gained after 24 h. Nevertheless, detection

of the sodorifen cluster mRNA using Northern blot was performed several times during this study, in contrast to transcriptome analysis, and always resulted in the same outcome. For this reason, it is most likely that expression of the sodorifen cluster reaches its maximum after 12-24 h cultivation, producing a large amount of protein whose activity is increasing throughout growth which results in the accumulating sodorifen amounts emitted by the cultures. Possibly, protein activity is modulated by precursors of the sodorifen biosynthesis which accumulate throughout growth and hence stimulate enzymatic conversion to sodorifen, e.g. by feed-forward induction.

Generally, the temporal regulation of sodorifen cluster expression was found to be equal in all producer strains tested and therefore is thought to represent a consistent regulatory mechanism among the different producer strains.

4.1.3 Promoter analysis

Since the sodorifen emission started at the transition from exponential to stationary phase growth going along with very high expression of the biosynthetic gene cluster, it was of interest to analyze the promoter region of the sodorifen cluster. In general, bacterial transcription is directed by DNA-dependent RNA polymerases (RNAP) which consist of four subunits (two α, β and β') to build the core enzyme which transcribes the DNA template into RNA. However, the core RNAP is not capable to identify the transcription initiation sites (i.e. promoters) alone. Only upon binding of a sigma (σ) factor, the holo-RNAP can locate the promoter region of a gene or cluster and initiate transcription (Paget and Helmann, 2003). In *E. coli* species, a total of seven different sigma factors could be identified which all lead to transcription of different sets of genes (Losick and Pero, 1981; Cook and Ussery, 2013). The primary sigma factor in all bacterial species is often referred to as σ^{70} (Gram-negative bacteria), σ^{A} (Gram-positive bacteria) or, more generally, the 'housekeeping' sigma factor since it is responsible for expression of genes necessary for vegetative growth. Likewise, certain stress conditions require activation of other genes, which are not expressed during vegetative growth, to cope with the respective stressor. For this purpose, bacteria employ alternative sigma factors. Accordingly, an increase in temperature demands expression of heat shock genes which preserve proteins from denaturation. Expression of these genes is directed by the σ^{32} (or σ^{H}) factor rather than σ^{70}. This 'division of labor' enables bacteria to switch gene expression patterns very fast, depending on the environmental conditions and thereby increases their survivability in nature.

If the feature of sodorifen emission would render its producers any survival benefit in nature, identification of the proper σ factor necessary for sodorifen biosynthesis could hint to its ecological function. Since binding of the appropriate sigma factor is

determined by the promoter sequence of the respective gene or operon, promoter analysis was performed for the sodorifen cluster. First, using 5'-RACE PCR the transcriptional start site of the sodorifen cluster was successfully determined 53 bp upstream of the IPP isomerase gene. Subsequent identification of the respective -10 and -35 regions which resemble the potential promoter region resulted in two possible sigma factors directing expression of the sodorifen cluster, namely σ^{70} and σ^{38} (or σ^{S}), since both share the same promoter recognition sequence (Espinosa-Urgel *et al.*, 1996; Ishihama, 2000; Lee and Gralla, 2001). Also, Gaal *et al.* (2001) proved binding of σ^{70} and σ^{S} to the same promoters *in vitro*, whereas *in vivo* promoters are specifically recognized by only one sigma factor. The promoter selectivity is, in this case, achieved by small alterations in the upstream sequence. For example, a TC motif at positions -14/-13 and an AT-rich region downstream of -10 favour a σ^{S} dependent transcription (Becker and Hengge-Aronis, 2001; Pruteanu and Hengge-Aronis, 2002). Also, σ^{70} favours spacer regions (nucleotides between the -10 and -35 region) with 17 bp in length. In contrast to this, Typas and Hengge (2006) reported that σ^{S} is capable of initiating transcription from promoters with varying spacer lengths ranging from 15 to 19 bp. The sodorifen cluster upstream sequence matches all of the aforementioned criteria including a shorter, 16 bp long spacer, already indicating a crucial role of σ^{S} in sodorifen biosynthesis.

Further indication was given by the fact that in contrast to σ^{70}, σ^{S} is responsible for expression of genes necessary for stationary phase rather than logarithmic growth (Mulvey and Loewen, 1989; Nguyen and Burgess, 1997). In agreement with this, sodorifen cluster transcription was proven to increase significantly during transition to stationary phase just like the sodorifen emission itself (Bernhardt, 2015). These findings are in contradiction with previous experiments where it was shown that slight expression of the sodorifen cluster was already detectable after only 5 h of growth, so during *lag* phase (Bernhardt, 2015). Nevertheless, expression level of the sodorifen cluster genes was significantly lower in comparison to later time points tested (e.g. 10 h). In general, RT-PCR resembles a very sensitive detection method, since present mRNA molecules are first converted in cDNA and afterwards repeatedly amplified. Therefore, it can be hypothesized that sodorifen cluster expression already takes place very early during bacterial growth but only to a very low extent resulting in equally low sodorifen biosynthesis and emission which might be below the detection limit of the VOC collection system or the GC/MS. From the literature it is known that sigma factors compete for a limited pool of RNA polymerase core enzymes (Ishihama, 2000). As a result, higher concentrations of a certain sigma factor increase the chance of binding to RNAP and therefore lead to the expression of the respective gene clusters. Using Western immunoblot analysis,' Jishage *et al.* (1996)

proved that σ^S concentrations in *E. coli* increase with time reaching its maximum in the stationary phase. But still, low amounts of σ^S were already detectable during earlier growth stages.

Therefore, it is reasonable to assume that small amounts of σ^S-bound RNAP (Eσ^S) are also present in logarithmically growing cells of *S. ply.* 4Rx13 leading to a low background expression of the sodorifen cluster which was detected by RT-PCR. Another possibility would be that sodorifen cluster expression is dependent on two sigma factors (σ^S and σ^{70}). On the one hand, both sigma factors share the same promoter recognition sequence and *in vitro* experiments proved equal expression levels by σ^S and σ^{70} of promoters containing 16 bp spacers, like in case of the sodorifen cluster (Typas and Hengge, 2006). On the other hand, if the sodorifen cluster transcription would also be dependent on σ^{70}, expression levels would be much higher during exponential growth than the ones detected by Northern blot.

Finally, a σ^S-negative mutant strain of *S. ply.* 4Rx13 was constructed which was no longer capable to emit sodorifen as proven by VOC analyses. In addition, expression of the sodorifen cluster was also shown to be completely absent, irrespective of higher amounts of RNA used for Northern blot, indicating the essential role of this sigma factor in sodorifen cluster transcription. Nevertheless, indirect effects of the performed knockout on expression of the sodorifen cluster can not be fully excluded since σ^S controls transcription of ca. 10 % of the *E. coli* genome (Weber *et al.*, 2005; Lacour and Landini, 2004; Patten *et al.*, 2004). An unequivocal proof could be given using EMSA (Electrophoretic Mobility Shift Assay) to demonstrate direct binding of σ^S to the promoter of the sodorifen cluster.

4.1.4 Stress-related function of sodorifen?

Notwithstanding, all results presented above confirmed the assumption that sodorifen cluster transcription is directed by the sigma S factor. This already indicated a potential role of sodorifen during stationary phase growth. However, σ^S is not only known for its involvement in stationary phase growth, but also in the general stress response of bacteria (Liu *et al.*, 2016; Battesti *et al.*, 2011). More specifically, the concentration of σ^S increases when the cells are subjected to different stress conditions so that σ^{70} can be replaced on the RNAP (Klauck and Hengge, 2012). The mechanism responsible for this global reaction to stress is called "cross-protection". For example, cells encountering carbon limitation also become more resistant to hydrogen peroxide, heat, osmotic stress and lower pH values (Jenkins *et al.*, 1988, 1990; Battesti *et al.*, 2011). To achieve this, using only one alternative sigma factor, σ^S itself is highly regulated on multiple levels. Not only can different stressors affect transcription and translation of *rpo*S (the gene encoding σ^S) but also the proteol-

ysis of the sigma factor itself and its capability to bind the RNAP core enzyme is regulated (Hengge, 2011). Several stress factors are also capable to interfere with gene expression, like osmotic pressure and low pH which both enhance translation of *rpo*S mRNA and, at the same time, hinder proteolysis of the newly synthesized sigma factor (Klauck and Hengge, 2012).

Since now σ^S is believed to activate sodorifen cluster expression, the effect of different stress conditions on the sodorifen emission was tested. Therefore, acidic as well as basic pH values, elevated sodium chloride concentrations and heat stress were applied to growing cultures of *S. ply.* 4Rx13. As a result, altering the pH value as well as osmotic stress reduced sodorifen emission in 4Rx13 significantly. Only using neutral pH values did not affect emission levels. This is in contrast to what was expected, because especially acidic pH values and high osmolarity should favour σ^S synthesis and stability (Lange and Hengge-Aronis, 1994; Muffler *et al.*, 1996; Bearson *et al.*, 1996; Sledjeski *et al.*, 1996; Majdalani *et al.*, 1998; Mandin and Gottesman, 2010; Bak *et al.*, 2014) and as a result also sodorifen emission. The primary cause for this discrepancy is most probably a result of the living cell numbers obtained upon stress application. Although experimental conditions were chosen to affect the living cell numbers of the stressed cultures as little as possible, alterations of bacterial growth could not be excluded completely. Especially in case of the hyperosmotic stress, living cell numbers were reduced by half due to salt application in comparison to the unstressed control. Lange and Hengge-Aronis (1994) already stated that high cell densities are crucial for induction of σ^S synthesis. More specifically, they stimulate transcription of the *rpo*S gene. As a result, reduced cell densities lead to low *rpo*S mRNA concentration which in turn removes the template for action of hyperosmotic and pH stress which both affect translation of the respective mRNA. To rule out low cell densities as the reason for low sodorifen emission, stressors could be applied later during growth, i.e. in stationary phase. Thereby, sufficient amounts of *rpo*S mRNA would already be present in the cells and the positive effect of hyperosmotic and low pH stress on translation of σ^S could emerge, ultimately leading to higher sodorifen cluster expression and emission. Surprisingly, low cell densities are not the reason for low sodorifen emission in case of acidic pH stress because living cell numbers were equally high in stressed and control cultures (results not shown). Until now, the reason for reduced sodorifen emissions, despite normal growth and the presumptive positive effect of acidic pH values on σ^S synthesis, is still not understood. Nevertheless, it can be speculated that the low pH may affect the bacterial metabolism in a way that precursors of the sodorifen biosynthesis are not produced. This idea is further supported by the fact that activity of the phosphofructokinase (PFK) is negatively affected at acidic pH values (Ugurbil *et al.*,

1978; Hayes *et al.*, 2006). PFK catalyses phosphorylation of fructose-6-phosphate which represents the rate-limiting step during glycolysis. Since sodorifen is thought to belong to the chemical class of sesquiterpenes, its biosynthesis is strongly dependent on availability of pyruvate and glyceraldehyde-3-phosphate, both deriving from glycolysis. Thus, low activity of PFK leads to reduced levels of the sodorifen precursors. Therefore, the positive effect of acidid pH values on σ^S translation and protein stability would indeed cause increased expression of the sodorifen cluster resulting in accumulation of sodorifen biosynthetic enzymes. Nonetheless, the lack of enzyme substrates would result in low sodorifen emission. Ultimately, it is also reasonable to believe that low pH values could also affect the activity of the sodorifen biosynthetic enzymes. To test this hypothesis, enzyme activity could be assessed at different pH values. Additionally, the analysis of sodorifen cluster expression and protein levels by Northern and Western blot, respectively, after cultivation of *S. ply.* 4Rx13 in low pH medium could give hints to possible transcriptional or translational actions of acidic pH on sodorifen emission.

As already mentioned above, additional to hyperosmotic and pH stress, also heat shock was applied to *S. ply.* 4Rx13 and its effect on sodorifen emission was investigated. It turned out that heat stressed cultures showed a slight increase of sodorifen emission in comparison to untreated controls of ca. 60 %. This is in good agreement with previous results obtained by Muffler *et al.* (1996) stating that a classical switch of the growth temperature from 30 °C to 42 °C increased stability of the σ^S factor in *E. coli*. Nevertheless, this sodorifen emission increase was not routinely observed in the biological replicates resulting in a high standard deviation. Possibly, the reason for these inconsistent results is preset by the experimental design. In the experiment performed in this study a classical heat shock was applied after 5 h of cultivation (OD_{600} = 0.1 $\pm$ 0.05, beginning of exponential phase) for 30 min. In contrast to this, different studies investigating the influence of heat stress on the σ^S factor were conducted in mid- or late-exponential phase for time periods ranging from 20 min to 6 h (Muffler *et al.*, 1996; Vidovic *et al.*, 2012). Also, just like hyperosmotic and acid stress, heat shock is known to affect protein stability of the σ^S factor positively. Therefore, it is reasonable to assume that later application of the stressor might result in a more significant and reproducible increase in sodorifen emission.

Taking all together, permanent stress conditions tested in this study (pH, salt) did not induce but significantly decreased sodorifen emission in *S. ply.* 4Rx13. On the other hand, application of transient stress (heat) seems to positively affect sodorifen emission. In order to assess if these effects are a consequence of transcriptional regulation, further investigations using Northern blot and RT-PCR are necessary. Nonetheless, according to the current results there is no indication that sodorifen

emission plays a role in stress defence. However, having in mind that these findings are based on experiments where stress was applied in very early growth stages, the results should be treated with considerable caution and experiments should be repeated with later application of stressors.

4.2 Effect of quorum sensing on the sodorifen emission in *Serratia* spp.

As already mentioned above, high cell densities are thought to enhance σ^S synthesis. Furthermore, sodorifen emission in *S. ply.* 4Rx13 starts to emerge at the beginning of stationary phase growth and thus also seems to require high cell numbers. A very prominent example of cell density dependent gene expression in bacteria is referred to as "quorum sensing" (QS). With this mechanism, microorganisms are capable of i) intercellular communication and ii) induction of multicellular behaviour by changing gene expression patterns (Fuqua *et al.*, 1994). The best studied example is the LuxI/LuxR system first identified in the symbiotic bacterium *Vibrio fischeri* leading to induction of bioluminescence (Hastings and Greenberg, 1999). In this case, the *lux*I gene encodes an acyl-homoserine lactone (AHL) synthase. The LuxI protein subsequently produces AHLs which serve as signaling molecules. Signal perception, in turn, is performed by the respective AHL receptor encoded by the *lux*R gene. At low cell densities, the amount of produced signaling molecules is not sufficient to activate LuxR. When cells start to multiply, also the AHL amount in the surrounding medium is increasing until a specific threshold concentration is reached and LuxR becomes activated. Finally, the AHL receptor can specifically recognize and bind to, so called, "lux-boxes" upstream of genes and operons regulated in response to cell density. In Gram-negative bacteria often homologues to the *Vibrio* LuxI/R system are found.

4.2.1 Identification of a potential quorum sensing system in *Serratia plymuthica* 4Rx13

Concerning *Serratia* spp. five different QS systems have been identified before, and were found to regulate various phenotypic traits like swarming motility, biofilm formation and antibiotic production (Eberl *et al.*, 1999; Thomson *et al.*, 2000; Horng *et al.*, 2002; Wei *et al.*, 2006). Therefore, it was reasonable to search the genome of *S. ply.* 4Rx13 for genes encoding potential quorum sensing systems. For the sodorifen producing strain *S. ply.* HRO-C48 Liu *et al.* (2007) already identified a functional quorum sensing system encoded by the genes *spl*I/R, which were used

as query sequences for *S. ply.* 4Rx13. As a result, two genes, annotated as *esa*l/R, were identified, matching the HRO-C48 query sequence to 99 % (on the amino acid as well as nucleotide level, alignments are presented in **supplemental figure S3**). Also, the convergent orientation of both genes was found to be identical to HRO-C48. In addition to the potential *spl*I/R system, two single LuxR-like proteins were identified in *S. ply.* 4Rx13 (ExpR, SolR) which had identities to SplR of 45 % and 31 %, respectively, but missed a corresponding LuxI-analogue.

Annotation of the potential QS genes, *esa*l/R, in *S. ply.* 4Rx13 leads to the assumption that they derived from the quorum sensing system of *Pantoea stewartii* ssp. *stewartii* (formerly classified as *Erwinia stewartii*). *P. stewartii* is the causative agent of Stewart's wilt disease in sweetcorn and leaf blight in maize (Minogue *et al.*, 2002). Plant infection is dependent on quorum sensing mechanisms performed by *esa*l/R (Coplin, 1992; Beck von Bodman and Farrand, 1995) with the special characteristic that EsaR, in contrast to other LuxR proteins, functions as a repressor rather than an activator of gene expression (von Bodman *et al.*, 1998). Nevertheless, comparison of amino acid sequences of the pantoean EsaI/R proteins revealed low identity to their potential equivalents in *S. ply.* 4Rx13 (EsaI 67 %; EsaR 80 %, **supplemental figure S4**). Thus, the QS system found in *S. ply.* 4Rx13 resembles the one present in its relative *S. ply.* HRO-C48 most, encoded by the genes *spl*I/R. As a consequence, re-annotation of *esa*l/R to *spl*I/R in *S. ply.* 4Rx13 is recommended but for reasons of consistency the naming of annotations *esa*l/R is kept throughout this thesis.

4.2.2 Role of *esa*l/R in sodorifen biosynthesis

After identification of a potential quorum sensing system, it was of great interest to assess its influence on sodorifen emission. First, using transcriptome data obtained previous to this study (Domik, 2015), expression of the quorum sensing genes *esa*l/R became obvious, implicating activity of quorum sensing in *S. ply.* 4Rx13. Subsequently, Teichmann (2016) proved that cell-free supernatants of *S. ply.* 4Rx13, applied to freshly inoculated cultures of the same species, induced sodorifen emission already during *lag* phase and more interestingly, increased sodorifen amounts by ca. 100 % in comparison to the control (culture without added supernatants). Quorum sensing signals are known to leave the producing cells by diffusion or advection and accumulate in the surrounding medium (Hense *et al.*, 2007). Therefore, it is reasonable to suspect that increase of sodorifen emission was achieved by N-acyl homoserine lactones present in the spent medium which was applied to the fresh culture of *S. ply.* 4Rx13.

To further substantiate the evidence that quorum sensing is involved in regulation

of sodorifen biosynthesis, insertional deletion mutagenesis was performed on *esa*I/R in *S. ply.* 4Rx13 leading to disparate results. Whereas dysfunction of *esa*R did not alter sodorifen emission, mutagenesis of *esa*I resulted in significant inhibition of the sodorifen emission by up to 90 %. Furthermore, Northern blot analysis revealed that reduced emission levels in *esa*I::Km is caused by decreased sodorifen cluster expression. The fact that mutagenesis of *esa*R did not affect sodorifen emission was rather surprising since it encodes the receptor which is thought to alter gene expression in response to quorum sensing signals produced by EsaI.

For *S. ply.* RVH1, van Houdt *et al.* (2007) reported that upon knockout of *spl*I AHL production was drastically reduced whereas in the *spl*R negative mutant it remained unaffected. Therefore, it can be reasoned that in *S. ply.* 4Rx13 knockout of *esa*R also does not alter AHL production. Whether these AHL's are capable of binding to another LuxR-like protein (i.e. ExpR or SolR) present in *S. ply.* 4Rx13, still remains a matter of speculation. From the transcriptome data, obtained for *S. ply.* 4Rx13, these two alternative LuxR-like proteins were shown to be expressed under sodorifen producing conditions just like for *esa*R, although their expression levels were much lower.

As already mentioned above, ExpR and SolR do not possess a cognate LuxI homologue. Therefore, they possibly constitute two examples of so-called "LuxR orphans" or "solos" (Fuqua, 2006; Patankar and González, 2009; Subramoni and Venturi, 2009) which is not unlikely since about 75 % of *lux*R genes are thought to encode such solos (Hudaiberdiev *et al.*, 2015). These LuxR orphans were first identified only recently and fulfil several functions in bacteria. First, detection of exogenous signals can be achieved using LuxR solos (i.e. signals produced by other species; Sperandio 2010; Soares and Ahmer 2011; Sabag-Daigle and Ahmer 2012). Second, they can also respond to signals other than AHLs (González and Venturi, 2013; Brachmann *et al.*, 2013; Brameyer *et al.*, 2014; Xu *et al.*, 2015; Brameyer *et al.*, 2015; Brameyer and Heermann, 2015; Chen *et al.*, 2015; Martínez *et al.*, 2015). Third and more importantly, unpaired LuxR proteins can be used to perceive endogenous signals, i.e. produced by the receiving organism itself (Chugani *et al.*, 2001; Lequette *et al.*, 2006). So far, the best studied example for the latter case is QscR from *Pseudomonas aeruginosa* which was not only shown to bind the endogenous QS signal (N-3-oxododecanoyl homoserine lactone, 3OC12-HSL) but also other long-chain AHLs and thus is thought to act in mixed species populations to integrate multispecies signaling (Lee *et al.*, 2006; Chugani and Greenberg, 2014).

Consequently, in case of the sodorifen biosynthesis in *S. ply.* 4Rx13, it can be suggested that upon inactivation of *esa*R, expression of either one ore both *lux*R solos is (are) activated which renders *S. ply.* 4Rx13 the possibility to complement the missing

EsaR receptor. Northern blot analysis of *expR* and *sol*R expression in the *esa*R::Km mutant could prove expression activation. Furthermore, construction of double or even triple mutants would eliminate the complementary effect and thus prove involvement of QS in sodorifen cluster expression. If complementation of EsaR by one or both LuxR solos could be proven in *S. ply.* 4Rx13, this would represent the first example of an unpaired LuxR gene which completely resembles/duplicates the function of another QS receptor, since QscR is known to respond to the same signal AHL but to cause a different transcriptional response in *P. aeruginosa* (Chugani *et al.*, 2001; Lee *et al.*, 2006).

As outlined above, first experiments strongly suggested a potential role of the EsaI/R QS system in sodorifen biosynthesis in *S. ply.* 4Rx13. Additionally, it is known that the genetically closely related strain *S. ply.* AS9 contains the sodorifen biosynthetic genes but does not emit sodorifen due to a lack of cluster expression. Furthermore, also differential expression and thus emission patterns were observed in the other sodorifen producing strains *S. ply.* HRO-C48, 3Re4-18, S13 and V4. One explanation for the low-/non-emission of sodorifen may be that there are differences in or complete absence of the quorum sensing system identified in *S. ply.* 4Rx13. For this purpose, genes homologous to *esa*l/R (present in *S. ply.* 4Rx13) were searched for in other *Serratia* spp. using BLAST analysis (Alikhan *et al.*, 2011). Thereby it became apparent that the sodorifen non-producing isolate AS9 is indeed missing a homologue to the QS signal synthase gene *esa*l. An *esa*R homologue could be determined, but only with an identity of 84 % to the gene present in *S. ply.* 4Rx13. Since knockout experiments in *S. ply.* 4Rx13 clearly showed that especially *esa*l has a positive influence on the sodorifen cluster expression, it can be assumed that a lack of AHL production causes the sodorifen-negative phenotype in AS9. On the other hand, BLAST analysis showed that the sodorifen producers *S. ply.* HRO-C48, 3Re4-18 and S13 do all possess a complete QS system with an identity of 99 % to the genes present in *S. ply.* 4Rx13, strengthening the evidence that QS is a prerequisite for sodorifen emission. However, the sodorifen producer *S. ply.* V4 was found to lack the *esa*l gene just as *S. ply.* AS9 did. So, according to the aforementioned hypothesis, *S. ply.* V4 should also be unable to emit sodorifen which was clearly not the case. Nevertheless, *S. ply.* V4 emitted the least sodorifen amounts among all sodorifen producing isolates identified so far. The same is true for *S. ply.* PRI-2C which previously was reported to be a sodorifen non-producer (Weise *et al.*, 2014). But more recent studies revealed that PRI-2C does indeed emit sodorifen but only to a very low extent (Schmidt *et al.*, 2017). Interestingly, also *S. ply.* PRI-2C was found to lack a homologue of the *esa*l gene. Consequently, it can be assumed that the *esa*l/R QS system does not activate, but rather increase sodorifen clus-

ter expression. This idea is further strengthened by the fact that mutagenesis of *esa*I in *S. ply.* 4Rx13 did not completely abolish sodorifen emission but only lead to a decreased emission level. Also, application of conditioned supernatants from *S. ply.* 4Rx13 on cultures of *S. ply.* AS9 did not induce sodorifen emission in this non-producing strain. Therefore, the lack of the AHL synthase, EsaI, alone can not be hold responsible for induction of sodorifen emission. Again, in the case of *S. ply.* AS9, it is most likely that deviances in the upstream sequence of the sodorifen cluster lead to non-induction of gene expression. Furthermore, also the other sodorifen producing strains HRO-C48, 3Re4-18 and S13, which were shown to contain a complete QS system equal to *S. ply.* 4Rx13, emitted only a fraction of the sodorifen amounts produced by *S. ply.* 4Rx13. Clearly, expression status of the *esa*I/R homologues in *S. ply.* HRO-C48, 3Re4-18 and S13 has to be investigated to ensure activity of quorum sensing in these organisms during sodorifen emission. Nevertheless, promoting effects of quorum sensing concerning sodorifen emission are in agreement with results obtained by application of conditioned medium (see above) where in *S. ply.* 4Rx13 an increase in sodorifen emission was detected whereas in *S. ply.* AS9 no positive effect was observed.

Furthermore, comparison of the nucleotide sequences also revealed other *Serratia* spp. possessing the same QS system like *S. ply.* 4Rx13 namely, *S. ply.* 3Rp8, RVH1 and G3. Especially the isolate *S. ply.* 3Rp8 seems to be interesting since BLAST analysis revealed that this strain also encodes a potential sodorifen cluster resembling the *S. ply.* 4Rx13 cluster to 98 %. Therefore, it can be hypothesized that *S. ply.* 3Rp8 represents a new member of sodorifen producing bacteria which needs to be further investigated by VOC analysis.

Nevertheless, the conclusion of a direct influence of quorum sensing on sodorifen biosynthesis should be treated with caution. Reduced expression of the sodorifen cluster upon deletion of the QS signal can also be attributed to indirect effects, due to a negative impact on another transcriptional regulator of sodorifen biosynthesis. In *Pseudomonas aeruginosa*, for example, 6-10 % of the genome is thought to be regulated in response to quorum sensing (Hentzer *et al.*, 2003; Schuster *et al.*, 2003; Wagner *et al.*, 2003). If the number of QS-regulated genes in *S. ply.* 4Rx13 is similarly high, the chance of indirect regulation increases.

So far it was also not possible to irrefutably identify binding sites for EsaR in the upstream sequence of the sodorifen cluster. In the literature, these binding sites are referred to as "lux boxes" or "lux box-types" (Devine *et al.*, 1988, 1989; Fuqua *et al.*, 1994; Whitehead, 2001). Unfortunately, only very degenerated consensus sequences have been published for these lux boxes in varying bacterial species, so far (Whiteley and Greenberg, 2001; Antunes *et al.*, 2008). Searching the sodo-

rifen cluster upstream region for these sequences revealed very high numbers of potential lux boxes making a clear identification, and moreover deletion, impossible. Nonetheless, using EMSA, binding of activated EsaR, ExpR and/or SolR could be demonstrated.

According to the results obtained in this study, sodorifen cluster expression is dependent on the σ^S factor. Therefore, indirect effects of QS on sodorifen emission are conceivable and have previously been demonstrated in *Pseudomonas aeruginosa* where *rpo*S transcription was shown to be regulated by the quorum sensing receptor LasR (Latifi *et al.*, 1996). In contrast to this, Schuster *et al.* (2004) reported that in *P. aeruginosa* ca. 18 % of genes, regulated by σ^S, were found to be co-regulated by quorum sensing. Therefore, it is possible that the observed reduction in sodorifen emission upon QS inactivation might be a result of reduced σ^S availability and thus cluster expression, as well as missing expression promotion by the QS receptor. However, in *E. coli*, no influence of quorum sensing on *rpo*S expression was detectable (Ihssen and Egli, 2004) strengthening the suggestion of direct involvement of EsaI/R in sodorifen regulation.

4.2.3 Identification of an AI-2 dependent quorum sensing system in *S. ply.* 4Rx13 and its effect on sodorifen emission

As already outlined above, quorum sensing is referred to as an intraspecific communication system to monitor population size and orchestrate collective behaviour. Nevertheless, also interspecific communication has been detected among bacteria (Bassler *et al.*, 1997) directed by the so-called autoinducer-2 (AI-2), a furanosyl borate diester (Schauder and Bassler, 2001; Federle and Bassler, 2003). Synthesis of this signaling molecule is achieved from S-adenosyl methionine (SAM) by a S-ribosylhomocysteine lyase, also referred to as LuxS (encoded by the *lux*S gene), by cleavage of S-ribosylhomocysteine to 4,5-dihydroxy 2,3-pentanedione (DPD) and homocysteine (Schauder *et al.*, 2001; Chen *et al.*, 2002). Since the *lux*S gene, and thus AI-2 itself, are highly conserved among Gram-negative and -positive bacterial species (Federle and Bassler, 2003; Vendeville *et al.*, 2005), it serves as the ideal cross-communication signal. Furthermore, AI-2 has been extensively studied concerning its role in biofilm formation (McNab *et al.*, 2003; Li *et al.*, 2007; He *et al.*, 2015, 2016; Ma *et al.*, 2017) and virulence factor production (Lyon *et al.*, 2001; Ohtani *et al.*, 2002; Winzer, 2002; Stroeher *et al.*, 2003; Kim *et al.*, 2003) in different species and more importantly, on secondary metabolite production in *Serratia* spp. (Coulthurst *et al.*, 2004). Here, reduced carbapenem production in *Serratia* ATCC 39006 and decreased prodigiosin synthesis in *Serratia marcescens* ATCC 274 was reported, upon marker exchange mutagenesis on the *lux*S gene (Coulthurst *et al.*,

2004). Therefore, it was interesting to investigate the role of a potential AI-2 dependent QS system on the sodorifen emission in *S. ply.* 4Rx13.

Using BLAST analysis and the *lux*S nucleotide sequence from *E. coli* K-12 MG1655 as query, a *lux*S homologue was successfully identified in *S. ply.* 4Rx13. A potential involvement of AI-2 in the regulation of sodorifen emission was further substantiated by the transcriptome data obtained for 4Rx13 under sodorifen producing conditions which revealed a very strong expression of the *lux*S gene (Domik, 2016, unpublished results).

Just like in the aforementioned LuxI/R systems, also AI-2 is sensed by a cognate receptor. In *Vibrio harveyi* signal perception is performed by LuxP in the periplasm which then modulates activity of a membrane-spanning sensor histidine kinase, LuxQ (Bassler *et al.*, 1994; Waters and Bassler, 2005; Pereira *et al.*, 2013). LuxPQ, in turn, evokes a signal transduction cascade by phosphorylation which finally leads to alteration of gene expression (Pereira *et al.*, 2013). So far, LuxP-like receptors were identified exclusively in bacteria of the genus *Vibrio* (Bassler *et al.*, 1994; Croxatto *et al.*, 2004; Sun *et al.*, 2004; Neiditch *et al.*, 2005, 2006; Rezzonico and Duffy, 2008).

In Gram-negative bacteria, different from *Vibrio* spp., AI-2 internalization is performed by proteins encoded by the *lsr* operon. Whereas LsrA/B/C/D function as a transporter of AI-2 across the membrane, LsrF/G are necessary for signal degradation inside the cell (Taga *et al.*, 2001, 2003; Miller *et al.*, 2004; Xavier and Bassler, 2005a,b; Pereira *et al.*, 2009; Torres-Escobar *et al.*, 2014; Marques *et al.*, 2014). In *E. coli*, *lsr*A-G are organized in a single operon which is, in absence of AI-2, under negative control of the repressor LsrR (Xavier *et al.*, 2007; Li *et al.*, 2007; Xue *et al.*, 2009). Therefore, prior to expression of the AI-2 transporter, the signal is thought to be imported into the cell by a PTS system (Pereira *et al.*, 2012) and is subsequently phosphorylated by the kinase LsrK (Xavier *et al.*, 2007; Pereira *et al.*, 2013; Zhu *et al.*, 2013). P-AI-2 can, in turn, relieve repression of the *lsr*A-G operon and exert its transcriptional control on its other target genes (Xavier *et al.*, 2007). Earlier analyses revealed that in *S. ply.* 4Rx13 an incomplete *lsr* operon is to be found (Teichmann, 2016) lacking the transcriptional repressor gene *lsr*R. Since expression of *lsr*A-G and *lsr*K could be detected by transcriptome analysis (Domik, 2016, unpublished results), AI-2 internalization and activation seems to take place under sodorifen producing conditions in *S. ply.* 4Rx13.

Unfortunately, insertional deletion mutagenesis on *lux*S did not markedly alter sodorifen emission. Although emission levels detected during 24-48 h of cultivation were slightly decreased, this result has to be treated with caution since the respective experiment was performed only once. Further biological replicates need

to be executed to finally exclude an involvement of AI-2 in sodorifen emission regulation. Additional construction of mutants defective in AI-2 internalization (*lsr*A-D) and, more important, AI-2 phosphorylation (*lsr*K) could give further evidence for an AI-2 independent regulatory system concerning sodorifen emission.

In conclusion, it can be reasonably hypothesized that quorum sensing mechanisms are involved in sodorifen cluster regulation. Specifically, an AHL-dependent system, consisting of the proteins EsaI/R, seems to play a crucial role in sodorifen regulation. But still, the effect of QS on sodorifen emission appears to be most pronounced in *S. ply.* 4Rx13 since in the other producing strains, which also possess a similar communication system, sodorifen emission remained low. In contrast to this, AI-2 dependent QS is suggested to not be involved in regulation of sodorifen biosynthesis.

4.3 Regulation of sodorifen emission by carbon catabolite repression

4.3.1 Influence of various carbon sources on sodorifen emission

As already stated above, sodorifen emission seems to be regulated both by differential mechanisms (among various producing strains, 4.1.1) as well as consistent regulatory processes (temporal regulation, 4.1.2). Another regulatory entity became apparent during feeding experiments of *S. ply.* 4Rx13, where different carbon sources were added to minimal cultivation medium (Magnus *et al.* 2017, von Reuß *et al.*, in preparation). These studies revealed a ca. 20-fold increase of sodorifen emission by *S. ply.* 4Rx13 upon addition of succinate as the sole carbon source.

Only recently, sodorifen was evidently characterized as a sesquiterpene, synthesized from MEP/DOXP-derived farnesylpyrophosphate and *S*-adenosyl methionine (von Reuß *et. al.*, in preparation). Therefore, initial substrates for sodorifen biosynthesis are glyceraldehyde 3-phosphate (G3P) and pyruvate reacting via the classic MEP/DOXP pathway to IPP which is then further converted into FPP. G3P and pyruvate can both derive from glycolysis and thus, glucose. As a consequence, glucose would be expected to serve as the perfect substrate for sodorifen emission. In contrast to this, it was highly surprising to see a strong inhibiting effect of glucose on sodorifen emission, amounting to almost 100 % (Magnus *et al.*, 2017).

Since application of succinate resulted in elevated sodorifen emissions and glucose caused reduced sodorifen detection, it was of interest to see the cumulative effect of both carbon sources in the same medium. As a result, very low sodorifen emission was detected which was almost equal to values obtained in glucose-only

medium. Additionally, different growth parameters (i.e. growth/division rate, doubling/generation time) of *S. ply.* 4Rx13 in MM + glucose, + succinate and + glucose/succinate were analyzed. Thereby it became apparent that there were no significant differences when comparing MM + glucose and MM + glucose/succinate. In contrast to this, using succinate as the sole carbon source resulted in decreased growth and division rates and consequently increased doubling and generation time. This would, at least in part, explain the sodorifen promoting effect of succinate since secondary metabolite production is known to be associated to low growth rates (Demain, 1989). Additionally, glucose consumption was monitored in MM + glucose in comparison to MM + glucose/succinate. As a result, glucose was removed from the mixed carbon culture much earlier than from the pure glucose culture. Most probably, this was due to growth limiting effects in the pure glucose culture where stationary phase was reached earlier than in the mixed glucose/succinate culture. This assumption can be further substantiated by results obtained from Weise (2013) who showed that in complex medium glucose is depleted within the first 24 h of cultivation resembling the result gained in the present study using MM + glucose/succinate.

Furthermore, applying different concentrations of glucose to the cultivation medium ranging from 10 mM to 100 mM led to a clear dose-dependent sodorifen inhibition (Weise, 2013; Magnus *et al.*, 2017). Moreover, sodorifen emission was less affected when glucose was added to complex medium rather than minimal medium (MM). This was probably due to the fact that complex medium still includes carbon sources different from pure glucose in contrast to the MM. Nevertheless, also in MM + glucose sodorifen emission was still observable, especially with low glucose concentrations. A possible explanation would be that low concentrations of the repressing carbon source lead to lower growth rates, which, in turn, favors secondary metabolite production (Demain, 1989; Ruiz *et al.*, 2010). Therefore, addition of low glucose concentrations would counterbalance its repressive effect on sodorifen emission. But against the supposition of low growth rates, no differences in living cell numbers have been observed in *S. ply.* 4Rx13 during cultivation in MM + glucose in comparison to MM + succinate, which is why growth impairment can be excluded to be the cause for the observed effect. Possibly, determination of different growth parameters (e.g. growth rate & doubling/generation time) would lead to a better understanding of the observed effect.

Irrespective of the reason for concentration dependent inhibition of sodorifen emission by glucose, expression analyses indicated that glucose-repression of sodorifen emission in *S. ply.* 4Rx13 is performed by transcriptional inhibition of the corresponding biosynthetic gene cluster, indicating involvement of regulatory processes.

Moreover, the same repressive effect of glucose on sodorifen emission was also

detected in the other producer strains *S. ply.* HRO-C48, 3Re4-18, S13 and V4, where a complete inhibition was observed. Likewise, addition of succinate as the sole carbon source caused an increase of sodorifen emission, just like in *S. ply.* 4Rx13, indicating that, in this regard, conserved regulatory mechanisms are manifested.

All the aforementioned results substantiate the hypothesis that glucose acts as a repressor on sodorifen emission and, more specifically, on sodorifen cluster transcription.

Especially in the genus *Streptomyces*, glucose-induced repression of secondary metabolite production has been studied extensively and was mostly related to a phenomenon referred to as carbon catabolite repression (e.g. formation of actinomycin Marshall *et al.* 1968; Gallo and Katz 1972; cephalosporin Aharonowitz and Demain 1978; actinorhodin and undecylprodigiosin Romero-Rodríguez *et al.* 2016).

Carbon catabolite repression (CCR) was initially identified as the mechanism behind hierarchical uptake preferences of different carbon sources. In a large number of cases, glucose is the preferred carbon source and causes CCR (Postma *et al.*, 1993; Kolb, 1993; Saier *et al.*, 1996). The general mechanism behind the phenomenon of CCR is known to act via a PTS depending system in Gram-negative bacteria (summarized in Ruiz *et al.* 2010).

The *E. coli* model system (summarized in Stülke and Hillen 1999; Deutscher 2008; Görke and Stülke 2008) comprises three high-energy phosphoprotein intermediates and five protein domains. The protein EIIA is phosphorylated by HPr, and transfers the phosphate to EIIB/C that reside at the cell membrane as a homodimer and then further to glucose rendering glucose-6-phosphate. Consequently, in the presence of glucose, EIIA is predominantly found in its dephosphorylated form, resulting in low adenylate cyclase activity. Since adenylate cyclase produces cAMP, high glucose levels result in low cAMP concentrations. Absence of cAMP, in turn, impedes complex formation with the cAMP receptor protein (CRP), which leads to non-activation of target operon expression. In contrast to this, low glucose levels result in accumulation of phosphorylated EIIA~P which activates adenylate cyclase. Consequently, cAMP levels increase and formation of the cAMP/CRP complex takes place. This activator complex can now bind to CRE sequences upstream of target operons and enhance their expression. Moreover, CCR was also proven to act by hindering other carbon sources to enter the cell (PTS-mediated inducer exclusion; Lengeler *et al.* 1998; Deutscher *et al.* 2006).

It is therefore reasonable to hypothesize that CCR is involved in the regulation of sodorifen production.

4.3.2 Mutagenesis of central carbon catabolite genes causes sodorifen emission reduction

To determine the role of CCR in the regulation of sodorifen biosynthesis, insertional deletion mutagenesis was performed on the central CCR genes: *cya* coding for the adenylate cyclase which is responsible for production of the second messenger cAMP and *crp* representing the cAMP receptor protein. As expected, both mutants showed an overall reduced sodorifen emission compared to the wild type, which already indicates an involvement of CCR in the regulation of sodorifen emission.

What was surprising was the fact that the *cya* mutant strain exhibited much higher sodorifen emissions than *crp*::Km. The reason for this rather contradictory result is still not entirely clear. The possibility that additional *cya* genes might be present in the genome of *S. ply.* 4Rx13 was ruled out since no homologous or annotated gene could be determined. Another assumption would be that beside cAMP also other nucleotides could bind to CRP leading to the formation of analogous complexes, which, in turn, can bind less efficiently to the sodorifen cluster upstream sequence. So far, several studies could show that variations in the amino acid sequence of CRP lead to either variations in ligand specificity or even to constitutively active CRP proteins (Garges and Adhya, 1985; Ryu *et al.*, 1993; Lee *et al.*, 1994; Youn *et al.*, 2006). Comparison of the amino acid sequences of the CRP protein in *E. coli* K-12 with *S. ply.* 4Rx13 revealed an identity of 99.52 % with a substitution at R123S. So far, nothing is known about the effect of such an alteration in the CRP protein and, to this point, it can only be speculated that it could lead to alteration in the CRP activity in *S. ply.* 4Rx13. Consequently, sodorifen emission would be observable in the *cya*::Km mutant in larger quantities than in the *crp*::Km mutant, where no binding to the upstream sequence of the sodorifen cluster happens anymore. Furthermore, Li *et al.* (2008) reported an effect of ATP on the antibiotic formation in *Streptomyces coelicolor*. During cultivation of this microorganism in the presence of 10 mM ATP, the actinorhodin concentration increased by 90 % compared to a culture grown in the absence of the nucleotide.

Another surprising result was the high sodorifen emission in *cya*::Km and *crp*::Km after 24 h cultivation in complex medium, which even exceeded that of the wild type. Northern blot analyses revealed that this can be attributed to an increased sodorifen cluster expression upon dysfunction of these two genes. However, Wang *et al.* (2005) could show that the *lux*S gene, which is responsible for production of the quorum sensing signal autoinducer-2 (for details see 4.2.3), is under negative control by cAMP/CRP. In an additional experiment, it was found that upon inactivation of *cya* and *crp* indeed the expression level of *lux*S was increased (**supplemental figure S5**). Therefore, AI-2 dependent quorum sensing could lead to induction of so-

dorifen cluster expression independent of CCR although mutagenesis of *lux*S itself did not alter sodorifen emission. As already indicated above, additional experiments need to be performed to substantiate the hypothesis of AI-2 involvement in sodorifen cluster regulation.

To further support the idea that the sodorifen cluster expression is regulated by CCR, two potential binding sites for the cAMP/CRP complex (CRE1/2) were identified in the upstream sequence of the sodorifen cluster. CRE motifs were, many times, shown to be essential for binding of the cAMP/CRP complex (e.g. in Villarreal *et al.* 2011; Herrera *et al.* 2012; Aung *et al.* 2014) using electrophoretic mobility shift assays (EMSA) and therefore provide evidence for a direct influence of carbon catabolite repression on operon expression.

Using homologous recombination, both CRE sequences were individually deleted from the upstream sequence in *S. ply.* 4Rx13. As a result, only deletion of CRE1 caused a significant reduction of the sodorifen emission and, to the same extent, cluster expression. On the other hand, deletion of CRE2, which is in closer proximity to the promoter region, showed no altered phenotype in comparison to the wild type. Therefore, it is legitimate to hypothesize that binding of cAMP/CRP only takes place at CRE1 upstream of the sodorifen cluster. Not just because of its higher identity to the consensus sequence and thus, higher affinity to the complex, but especially because of its positive effect on the sodorifen production. Nevertheless, also simultaneous binding of two cAMP/CRP complexes, to CRE1 and CRE2, respectively, would be possible. For example, it was shown for the *E. coli deoP2* promoter that binding of two cAMP/CRP complexes to two distinct CRE sites is necessary for the cytidine repressor (CytR) to bind and suppress expression of catabolic genes for nucleosides (Søgaard-Andersen *et al.*, 1990; Valentin-Hansen *et al.*, 1996; Chahla *et al.*, 2003). In this case, the two CRE sites exhibit different affinities for the cAMP/CRP complex with the most upstream one having the highest. Binding of one or two cAMP/CRP complexes enhances expression of the following operon, whereas occupation of both CRE sites also renders the opportunity for the CytR repressor to bind and stop expression. Therefore, in case of the sodorifen cluster, a similar system might be active. Consequently, CRE1 would act as binding site for enhancing sodorifen cluster expression, whereas occupancy of CRE2 would lead to recruitment of an additional repressor. As a result, deletion of CRE2 would have no repressive effect on the sodorifen emission, in contrast to CRE1. To test this hypothesis, identification of possible repressors and prove of cAMP/CRP binding to the potential CRE sites will be necessary. Moreover, it was interesting to see that particularly the CRE2 sequence was completely missing in the sodorifen-negative strain *S. ply.* AS9, whereas for CRE1 only three nucleotide exchanges were observed.

Kurrer (2017) successfully replaced the 3 mismatches in *S. ply.* AS9 with the ones present in *S. ply.* 4Rx13 which did not transform *S. ply.* AS9 into a sodorifen producing strain. Therefore, a role of CRE1 as a crucial determinant for a bacterium to emit sodorifen can be excluded, which suggests that additional factors are involved in transcription initiation at the sodorifen cluster. It will be of great interest in the future, to exchange the *S. ply.* AS9 upstream sequence, in part or completely, with the upstream sequence from *S. ply.* 4Rx13, to check if only the lack of binding sites for transcriptional inducers, e.g. of cAMP/CRP or others, that have not been identified so far, is responsible for its non-sodorifen-producing phenotype.

Just as in the case of quorum sensing, indirect involvement of CCR in sodorifen regulation is possible. Likewise, cAMP/CRP, the central effector complex of the carbon catabolite repression, is known to positively affect *rpo*S transcription during late-exponential growth phase in *E. coli* (Mika and Hengge, 2005). Anyhow, the negative effect of CRE1 deletion on the sodorifen emission in *S. ply.* 4Rx13 clearly showed that carbon catabolite repression is directly involved in regulation of the sodorifen biosynthesis.

5 Conclusion

The function of the volatile sesquiterpene sodorifen has been a hot research topic for several years. So far, no ecological relevance of this unique molecule could be identified although it is produced in such high amounts. Therefore, it was the aim of this study to identify regulatory mechanisms controlling sodorifen emission and biosynthesis in *Serratia* spp. to be finally able to draw conclusions towards its biological role.

Generally, it can be stated that sodorifen emission is regulated, like most secondary metabolites, in response to growth rate. More specifically, it became apparent that another regulatory level of sodorifen emission is targeting transcription of the sodorifen biosynthetic gene cluster. The first and most important regulatory entity was found to be the σ^S dependent transcription initiation. Since dysfunction of the promoter-recognizing RNAP subunit resulted in complete loss of sodorifen cluster transcription, σ^S is considered to be compulsory for the feature of sodorifen production. Interestingly, involvement of this sigma factor can be used as an indication for a stress-related function of sodorifen due to the fact that σ^S is one of the central key-regulators in the bacterial stress response.

Secondly, transcriptional regulation occurred via quorum sensing using an AHL-dependent mechanism. To date, it is not known which quorum sensing signal is responsible for induction of sodorifen emission, but experiments, involving conditioned medium and QS-negative mutants, strongly suggest further activation of sodorifen cluster expression in a cell number dependent manner.

The third main regulatory unit was identified by previous experiments which showed that sodorifen emission varies significantly in the same organism depending on the carbon source used (Magnus *et al.* 2017, von Reuß *et al.*, in preparation). Thereby, glucose was identified as a repressor of sodorifen emission and likewise cluster expression in *S. ply.* 4Rx13 and also in all other producing strains identified so far, indicating a potential role of carbon catabolite repression in sodorifen regulation. Regarding the results obtained from knockout mutants of central CCR genes it was concluded that sodorifen cluster expression is positively controlled by this global regulatory mechanism.

Nevertheless, the possibility exists that sodorifen emission is not directly controlled by quorum sensing or carbon catabolite repression. Since RpoS (σ^S) was found to be essential for sodorifen cluster expression, effectors acting on the expression or activity of this protein, could indirectly influence sodorifen cluster expression. Accordingly, quorum sensing and carbon catabolite repression are both known to influ-

ence σ^S activity and therefore can indirectly affect sodorifen emission.

Additionally, regulation of sodorifen biosynthesis is assumed to proceed also via post-transcriptional control. Indications were given for example by reduced sodorifen emission levels detected in *S. ply.* 4Rx13 upon cultivation in MM + succinate and methionine which were shown to be not related to reduced cluster expression. The mechanism(s) by which this post-transcriptional regulation is performed is (are) still not understood.

Regarding all aforementioned results, it is suggested that sodorifen emission is controlled by several global regulatory processes, as summarized in **figure 5.1**. σ^S is thought to be the main factor leading to activation of sodorifen cluster expression. Upon transcriptional induction, additional effectors can further enhance expression of the cluster genes, e.g. by binding of an activated quorum sensing receptor or the cAMP/CRP complex to specific operator sequences upstream of the promoter.

Concerning the ecological function of sodorifen it can be speculated that its emission renders a survival benefit to the producing organism, especially concerning growth under various stress conditions.

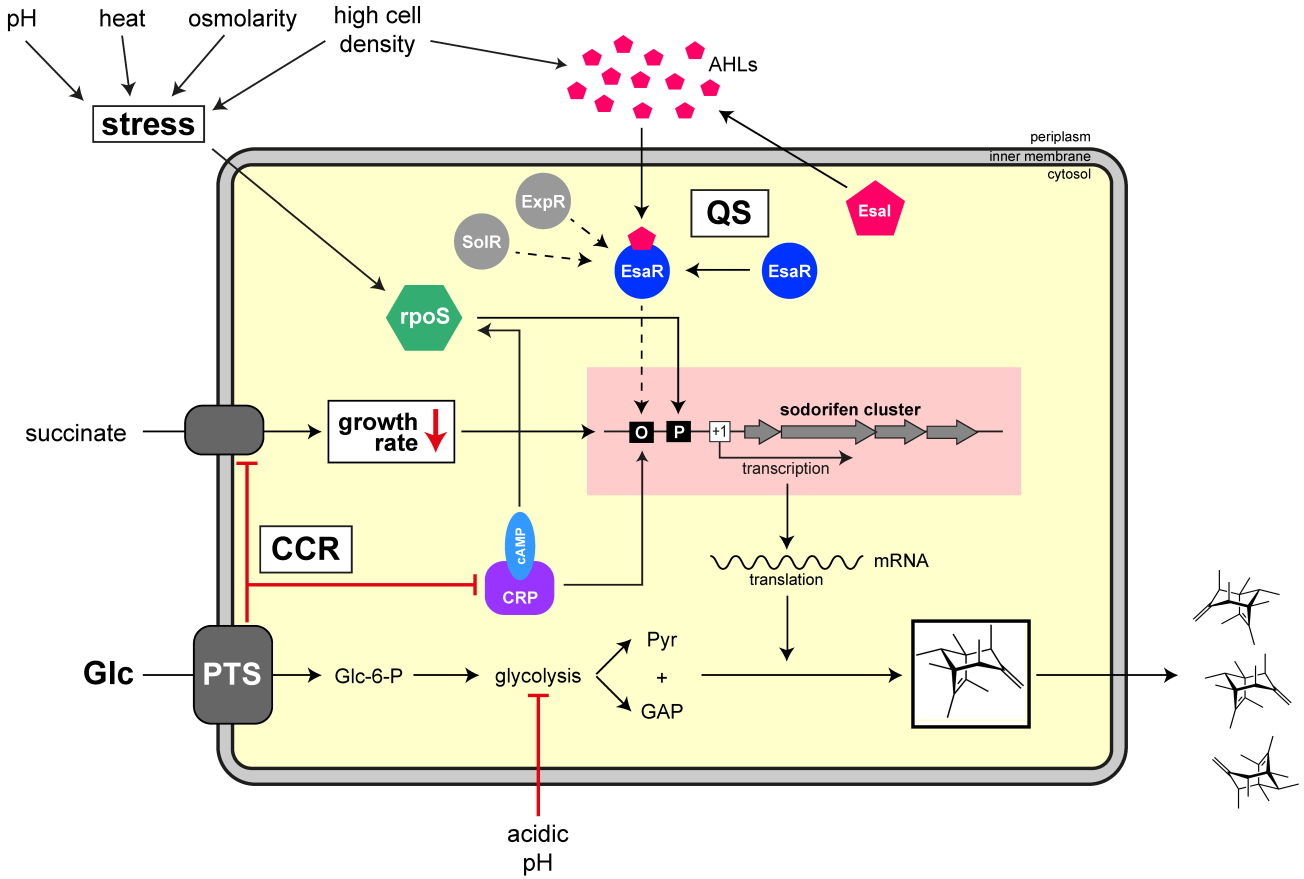

Figure 5.1: Regulation of sodorifen emission. The main regulatory entities involved in sodorifen emission control are represented in white boxes. For mechanistic details see chapter 5. Black arrows indicate positive effects elicited by the respective mechanisms. Dashed lines illustrate hypothetical effects where final evidence is missing. Blocked, red lines represent inhibition. QS = quorum sensing, CCR = carbon catabolite repression, O = operator binding site, P = promoter, +1 = transcriptional start site, Glc = glucose, Glc-6-P = glucose-6-phosphate, Pyr = pyruvate, GAP = glyceraldehyde-3-phosphate, PTS = phosphotransferase system.

6 Further perspectives

To further substantiate the assumption of a stress-related function of sodorifen, additional stress experiments have to be performed including also biotic stress (e.g. by fungi). In case of *S. ply.* PRI-2C, induction of sodorifen emission due to exposure to volatiles produced by the fungus *Fusarium culmorum* has already been shown previously (Schmidt *et al.*, 2017). Since PRI-2C is known to produce only very low amounts of sodorifen it will be intriguing to investigate the influence of fungal VOCs on *S. ply.* 4Rx13. Furthermore, stress experiments which were already performed, dealing with abiotic stress, did not reveal any influence on sodorifen emission. For this reason, these studies will be repeated changing the time point, as well as duration of stress application. Since sodorifen emission is dependent on σ^S, which is produced upon entry of the bacterial cells into stationary phase, stress factors will be applied at this particular growth stage, to possibly see an enhanced sodorifen emission, as initially expected.

Since some experiments indicated the involvement of post-transcriptional regulation, it would be helpful to determine the amount of the sodorifen biosynthetic enzymes after cultivation under the corresponding conditions using Western blot.

Furthermore, focus should be put on evaluation of the enzymatic activity of the sodorifen cluster proteins under different conditions, e.g. pH alterations and osmotic stress, to identify potential post-translational regulatory mechanisms.

The genetically close relative *S. ply.* AS9 contains the sodorifen cluster, which was shown to be not expressed in the respective strain. Probably, this is due to differences in the upstream sequence of *S. ply.* AS9, in comparison to *S. ply.* 4Rx13. Consequently, it will be of interest to exchange the upstream sequence in the sodorifen non-producer *S. ply.* AS9 with the one present in the producer strain *S. ply.* 4Rx13 to potentially induce cluster expression and thus sodorifen emission. Furthermore, also the other sodorifen producing strains *S. ply.* HRO-C48, 3Re4-18, S13 and V4 showed reduced expression of the sodorifen cluster genes. To assess the possibility of promoter shifts in these organisms, 5'-RACE-PCR will be performed for determination of the transcriptional start site and thus promoter region of the sodorifen cluster. The same should also be applied to the non-producer *S. ply.* AS9.

At last, binding of potential transcription factors to the upstream sequence of *S. ply.* 4Rx13 needs to be proved, using for example the electrophoretic mobility shift assay. Therefore, binding of σ^S, cAMP/CRP and EsaR could be irrefutably demonstrated.

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8 Supplemental information

8.1 Primer

All primers used during this work are listed in table S1.

Table S1: Primers used for PCR reactions. If not stated otherwise, the presented primers were designed for use in *Serratia plymuthica* 4Rx13. Underlined nucleotides in primer sequences represent the part binding to the target DNA and were used for calculation of T_m.

Name	Purpose	Direction	T_m (°C)	Sequence (5' → 3')
1.5	Detection of FRT-PGK-gb2-neo-FRT cassette	sense	68.6	CCAAGGCAGTCTGGAGCATG
1.6		antisense	68.7	GGGAGCTCTCAGACGTCGCT
62.1	Detection of *crp*	sense	70.8	TCTCGGCAAACCGCAAACAG
62.2		antisense	67.1	CGTGAACAACCGACGATCTG
62.3	Mutagenesis of *crp*	sense	67	GCTCCGTCGCGGTGCTGATTAAAG ATGAAGAGGGTAAAGAGATGATCC TGAATTAACCCTCACTAAAGGGCGG
62.4		antisense	61.5	CTGCGCTCCTGGCCTTCTTCAAAC AATCCAAGCTCGCCGATGAAATCC CCTAATACGACTCACTATAGGGCTC
63.1	Detection of *cya*	sense	65.1	GAGACTGGATGCGATCAACC
63.2		antisense	67.1	CCCGTTCGATCTTCCAGTTG
63.3	Mutagenesis of *cya*	sense	67	CCCGCGTTTCAACGGGTATACAGT CTGCTGCCTACCTTACTGCACTAC CAAATTAACCCTCACTAAAGGGCGG
63.4		antisense	61.5	CAGGCGTGTAAAGGCAAACGCCG TGGGGAACGTTACCGTTCAGATAG CCCTAATACGACTCACTATAGGGCTC
71.1	Amplification of terpene cyclase	sense	65.4	ATCCCTTCAACCGGCAGTAC
71.2		antisense	64.1	CTGAAGCGGTATAAGCCAGC
72.1	Amplification of methyltransferase	sense	62	CCGACAGCTATGAGGAGAAG
72.2		antisense	66.1	GCAGGCAATCACGCTGTAAG
73.1	Amplification of DOXP synthase	sense	67.8	GCGGAAATTAACCCGTGAGC
73.2		antisense	68.1	CCACGTCGGCAACAAACTTC
74.1	Amplification of IPP isomerase	sense	64.7	ACAAAGGAAGATGGCTGCTG
74.2		antisense	62.8	CAGGCTCACCTTCAAACAAC

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Table S1: Primers used for PCR reactions. If not stated otherwise, the presented primers were designed for use in *Serratia plymuthica* 4Rx13. Underlined nucleotides in primer sequences represent the part binding to the target DNA and were used for calculation of T_m.

Name	Purpose	Direction	T_m (°C)	Sequence (5' → 3')
85.1	Amplification of *esa*I	sense	66.3	GAAAGTGACGCACTCGAACG
85.2		antisense	65.1	GACGATCTCGGACAACCAAG
85.3	Mutagenesis of *esa*I	sense	67	TCAGTGACCGTCTGGGGTGGGACG TAGCATGCAACCTCGACATGGAGT TTAATTAACCCTCACTAAAGGGCGG
85.4		antisense	61.5	CAAACCAACTGTCCTTGGCATAGA CCCCCGCACACGTTACATTCTCGG TTTAATACGACTCACTATAGGGCTC
86.1	Amplification of *esa*R	sense	64.7	CACTTCGGGACCATATCGAC
86.2		antisense	64.8	GTCGCTGCCGGTGTAATAAG
86.3	Mutagenesis of *esa*R	sense	67	CATCAGATGTACTTATTATTTCAA GTTACCCGGATGAATGGGTTGATC TTAATTAACCCTCACTAAAGGGCGG
86.4		antisense	61.5	GACGTTCGTTTGAACGCAGTCAGT TTTCCAGCTCACTGACCCGGTGAT TTTAATACGACTCACTATAGGGCTC
89.1	Amplification of sodorifen cluster upstream sequence	sense	64.6	GATCCATCCGATTTGACACC
89.2		antisense	65.1	ATTCGATTGAGCATTGGGTG
89.3	Mutagenesis of CRE1	sense	67	GGGTATCGGTGAGCACGTAATATC ATTGGGCTAGCCGACCCCTTTGTT TCAATTAACCCTCACTAAAGGGCGG
89.4		antisense	61.5	TGGGAGTTTTGCTAGGGTGCAACT ATTTTTTGTTAAAGCCAGGCGATG GATAATACGACTCACTATAGGGCTC
90.3	Mutagenesis of CRE2	sense	67	ATTAAGTTAAACCATAAGTTAAAA ATATATCACTCGCTGATACTTCAT CAAATTAACCCTCACTAAAGGGCGG
90.4		antisense	61.5	TGTCAAATTTTATTTAATGACGGT TTTTAAGAAAAATAAATTAATCAT GATAATACGACTCACTATAGGGCTC
102.1	Detection of *rpo*S	sense	69.8	GGATGATGCGGATTTCGACG
102.2		antisense	67.5	TCTCTGAATCACCGCCCAAC

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Table S1: Primers used for PCR reactions. If not stated otherwise, the presented primers were designed for use in *Serratia plymuthica* 4Rx13. Underlined nucleotides in primer sequences represent the part binding to the target DNA and were used for calculation of T_m.

Name	Purpose	Direction	T_m (°C)	Sequence (5' → 3')
102.3	Mutagenesis of *rpo*S	sense	67	GAATGATCGAAAGCAACCTGCGGC TGGTGGTGAAAATTGCCCGTCGCT ACAATTAACCCTCACTAAAGGGCGG
102.4		antisense	61.5	ACTGCACGGATCAGGCCGAGGTTG CCCTCTTCAATCAGATCCAGCAGC GCTAATACGACTCACTATAGGGCTC
16 S rRNA fwd	Amplification of 16 S rRNA	sense	62	AGAGTTTGATCATGGCTCAGA
16 S rRNA rev		antisense	70	AGGTGATCCAACCGCAGGTTC
Q_O	5'-RACE-PCR	sense	61.5	CCAGTGAGCAGAGTGACG
Q_i		sense	61	GAGGACTCGAGCTCAAGC
Q_T		sense	48	CCAGTGAGCAGAGTGACGAGGACTC GAGCTCAAGCTTTTTTTTTTTTTTTT
GSP1		antisense	69.1	CTTCCTGTAAACGCCGCTGG
GSP2		antisense	72.8	ATGGCCGCAGCAACTGTTGG

8.2 5'-RACE

The protocol used for 5'-RACE-PCR was adapted from the publication of Scotto-Lavino *et al.* (2006) and is presented in **table S2**.

Table S2: Protocol for 5'-RACE-PCR. Modified after Scotto-Lavino *et al.* (2006). The sequences of primers used can be found in supplemental table S1.

Destruction of RNA template	
RNaseH (2 U/µl; **FIRMA**)	0.75 µl
37 °C, 20 min	
ad. 400 µl with TE buffer (10 mM Tris base, 1 mM EDTA), 4 °C	
Clean-up of PCR product (see 2.3.8)	
Elution in 15 µl of ddH_2O	
Poly-(A)-tailing	

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Table S2: Protocol for 5'-RACE-PCR. Modified after Scotto-Lavino *et al.* (2006). The sequences of primers used can be found in supplemental table S1.

5x Reaction buffer for Tdt	4 µl
dATP (1 mM)	4 µl
Terminal Deoxynucleotidyl Transferase (Tdt, 20 U/µl, Thermo Fisher Scientific, Waltham, USA)	1.5 µl
15 min, 37 °C; 10 min, 70 °C ad. 500 µl with TE buffer, 4 °C	
1st round of amplification	
5x HF buffer (Green)	10 µl
dNTPs (10 mM each)	1 µl
5' end tailed cDNA (from previous step)	1 µl
Primer (10 pmol/µl) (GSP1,Q_O, Q_T)	2.5 µl
Phusion HS II polymerase (Thermo Fisher Scientific, Waltham, USA)	1.25 µl
ddH_2O	29.25 µl
5 min, 98 °C; 2 min, 48 °C; 40 min, 72 °C **30 cycles of:** 10 sec, 98 °C; 10 sec, 61.5 °C; 1.5 min, 72 °C 10 min, 72 °C, ∞, 10 °C	
2nd round of amplification	
5x HF buffer (Green)	10 µl
dNTPs (10 mM each)	1 µl
Amplification product from 1st round	1 µl
Primer (10 pmol/µl) (GSP2,Q_i)	2.5 µl
Phusion HS II polymerase (Thermo Fisher Scientific, Waltham, USA)	1.25 µl
ddH_2O	31.75 µl
5 min, 98 °C **30 cycles of:** 10 sec, 98 °C; 10 sec, 61 °C; 1.5 min, 72 °C 10 min, 72 °C, ∞, 10 °C	
Clean-up of final PCR product (see 2.3.8)	

8.3 Sodorifen cluster upstream sequence alignment - summary

Table S3: Comparison of sodorifen cluster upstream sequence from *S. ply.* 4Rx13 with HRO-C48, S13, V4 and AS9. Alignments were conducted using the Clustal Omega online tool (McWilliam *et al.*, 2013). The upstream sequence of the sodorifen cluster in *S. ply.* 4Rx13 was used as a reference for identity calculation.

Strain	Total identity	Alterations in nucleotide sequence in comparison to *S. ply.* 4Rx13
HRO-C48	96.25 %	14A>G; 176T>C; 197C>T; 318T>C; 319C>A; 368C>A; 375A>G; 378G>A; 410T>G; 435T>C; 481_488insTGGAAGAA
3Re4-18	97.71 %	14A>G; 176T>C; 197C>T; 318T>C; 319C>A; 368C>A; 375A>G; 378G>A; 410T>G; 435T>C; 480del
S13	96.25 %	14A>G; 176T>C; 197C>T; 318T>C; 319C>A; 368C>A; 375A>G; 378G>A; 410T>G; 435T>C; 481_488insTGGAAGAA
V4	94.38 %	14A>G; 30T>C; 111T>A; 149_150insG; 176T>C; 185G>A; 197C>T; 213G>A; 218C>T; 221C>T; 226G>A; 236A>G; 240A>T; 250G>T; 252A>G; 255C>A; 318T>C; 319C>A; 378G>A; 391A>G; 405G>A; 410T>G; 435T>C; 447A>T; 450G>C; 451C>T; 452T>G; 480del
AS9	74.79 %	> 100

8.4 Effect of succinate and methionine on sodorifen production

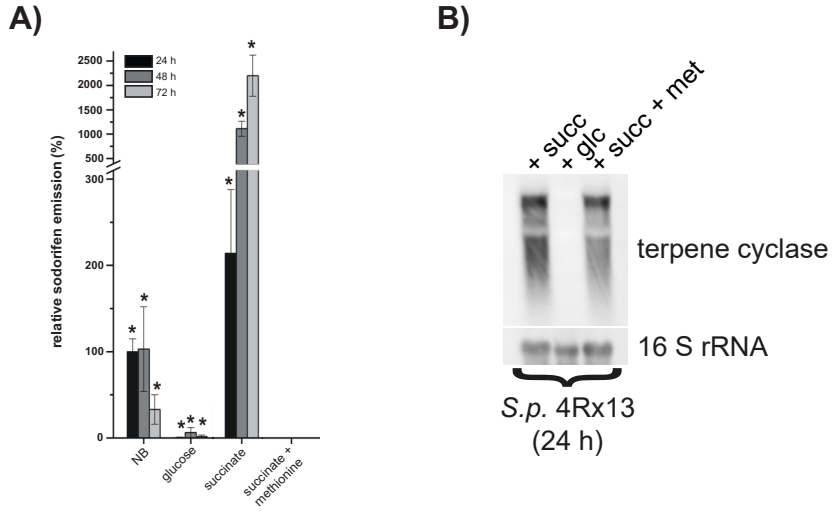

Figure S1: Effect of succinate and methionine on sodorifen production. A) Relative sodorifen emission of *S. ply.* 4Rx13 during cultivation in complex medium (NB) or minimal medium supplemented with 55 mM glucose (glc), succinate (succ) or succinate + 20 mM methionine (succ + met). Sodorifen emission was detected using SPME followed by GC/MS analysis. For quantification, sodorifen emission was calculated relative to the peakarea of sodorifen after 24 h cultivation in complex medium (100 %). Modified after Weise (2013). **B)** Expression levels of the terpene cyclase in *S. ply.* 4Rx13. Northern blot was performed using 5 µg RNA after 24 h cultivation in the respective media. A DIG-dUTP labelled probe specific for the terpene cyclase mRNA was used. As a control a labelled probe detecting the 16 S rRNA was applied afterwards. Probe hybridiziations were visualized by fluorenscence measurements for 3 min.

8.5 Effect of different amino acid combinations on sodorifen cluster expression

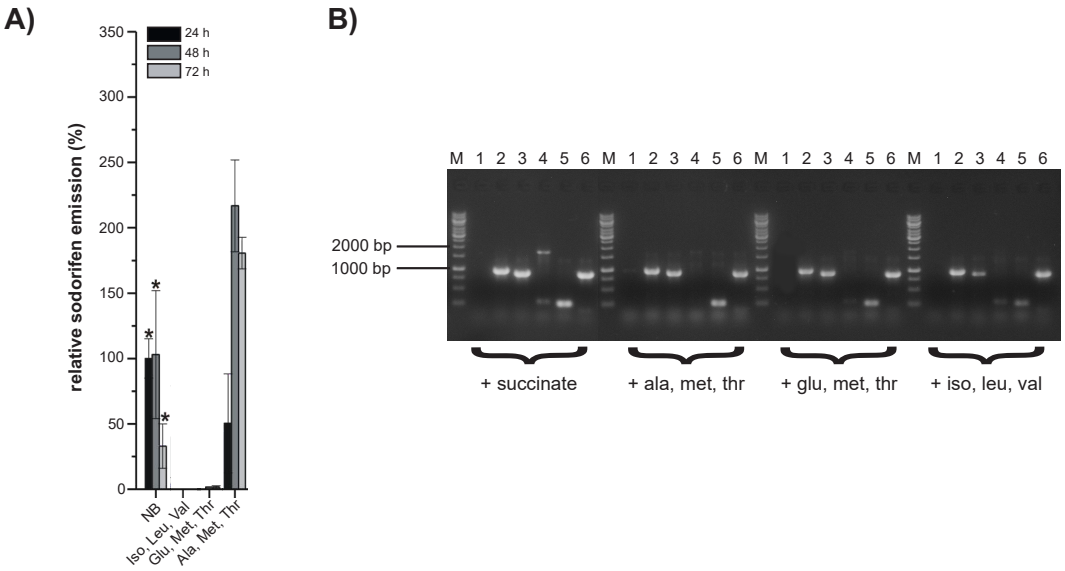

Figure S2: Effect of different amino acids on sodorifen cluster expression in *S. ply.* 4Rx13. A) Relative sodorifen emission of *S. ply.* 4Rx13 during cultivation in complex medium (NB) or minimal medium supplemented with different amino acid combinations (20 mM each). Sodorifen emission was detected using SPME followed by GC/MS analysis. For quantification, sodorifen emission was calculated relative to the peakarea of sodorifen after 24 h cultivation in complex medium (100 %). Modified after Weise (2013). **B)** RT-PCR of the sodorifen cluster genes. Lane 1 = negative control (no reverse transcriptase), lane 2=terpene cyclase, lane 3 = methyltransferase, lane 4 = DOXP synthase, lane 5 = IPP isomerase, lane 6 = positive control (GAP-DH). M= marker (GeneRuler 1 kb, Thermo Fisher Scientific, Madison, USA). Isoleucine (Iso), leucine (Leu), valine (Val), glutamic acid (Glu), methionine (Met), threonine (Thr), alanine (Ala).

8.6 Alignment of quorum sensing proteins

ALIGNMENT
Serratia plymuthica HRO-C48 SplI vs. *Serratia plymuthica* 4Rx13 EsaI

Score = 434 bits (1115), Expect = 2e-158, Method: Compositional matrix adjust.
Identities = 208/210 (99%), Positives = 209/210 (99%), Gaps = 0/210 (0%)

```
HRO-C48 - SplI    1    MLELFDVSYEELKVTHSNELYRLRKKTFSDRLGWDVACNLDMEFDEFDNPRTRYILGLCQ  60
4Rx13 - EsaI      1    ............................................................  60

HRO-C48 - SplI    61   GQLVCSVRFIALSQPNMITHTFCSCFDSVHIPFEGIESSRFFVDKARARQLLGEQYPVSQ  120
4Rx13 - EsaI      61   ............................................................  120

HRO-C48 - SplI    121  ALFLAMINWGHHHGRNGIHTIVSRAMLRILQRSGWKISVLKEAFLTERERIYLVFLPTGS  180
4Rx13 - EsaI      121  ............E...............................................  180

HRO-C48 - SplI    181  EDQNRVASGIVTGLGCPRSSVITWPLSLPV  210
4Rx13 - EsaI      181  .....M......................... 210
```

ALIGNMENT
Serratia plymuthica HRO-C48 SplR vs. *Serratia plymuthica* 4Rx13 EsaR

Score = 511 bits (1317), Expect = 0.0, Method: Compositional matrix adjust.
Identities = 247/249 (99%), Positives = 248/249 (99%), Gaps = 0/249 (0%)

```
HRO-C48 - SplR    1    MFSVFNKNQTITETLRDHIDRKLSSLGSPEYAYTVVRKKNPSDVLIISSYPDEWVDLYCA  60
4Rx13 - EsaR      1    ............................................................R.  60

HRO-C48 - SplR    61   NNFQLTDPVILTAFKRTSPFAWDENITLMSDLKFTKIFSLSKQYNIINGFTFVLHDHMNN  120
4Rx13 - EsaR      61   ............................................................  120

HRO-C48 - SplR    121  LALLSVIIASNGQEELEKRLSSERGAMQMLLIDINEQMYRLAGAASSSNQKYHTDADKPI  180
4Rx13 - EsaR      121  .........N..................................................  180

HRO-C48 - SplR    181  FTPRENEVLYWASMGKTYGEIAAIAGISVSTVKFHMGNIVVKLGVSNARQAIRLGVELEL  240
4Rx13 - EsaR      181  ............................................................  240

HRO-C48 - SplR    241  ITPAATAAR  249
4Rx13 - EsaR      241  .........  249
```

Figure S3: Alignment of SplI/R (*S. ply.* HRO-C48) with EsaI/R (*S. ply.* 4Rx13. Alignment was performed using Protein BLAST analysis (NCBI, Altschul *et al.* 1990). Deviating amino acids are indicated in bold red letters. Matches are represented by dots.

```
ALIGNMENTS
>WP_004950753.1 acyl-homoserine-lactone synthase [Serratia plymuthica]
 AGO53109.1 acyl-homoserine-lactone synthase EsaI [Serratia plymuthica 4Rx13]
Length=210

 Score = 306 bits (783),  Expect = 8e-108, Method: Compositional matrix adjust.
 Identities = 141/210 (67%), Positives = 168/210 (80%), Gaps = 0/210 (0%)

Pantoea - EsaR  1    MLELFDVSYEELQTTRSEELYKLRKKTFSDRLGWEVICSQGMESDEFDGPGTRYILGICE  60
4Rx13   - EsaR  1    ............KV.H.N...R............D.A.NLD..F....N.R......L.Q  60

Pantoea - EsaR  61   GQLVCSVRFTSLDRPNMITHTFQHCFSDVTLPAYGTESSRFFVDKARARALLGEHYPISQ  120
4Rx13   - EsaR  61   .........IA.SQ........CS..DS.HI.FE.I.............Q....Q..V..  120

Pantoea - EsaR  121  VLFLAMVNWAQNNAYGNIYTIVSRAMLKILTRSGWQIKVIKEAFLTEKERIYLLTLPAGQ  180
4Rx13   - EsaR  121  A.....I..GHHHERNG.H........R..Q....K.S.L.......R.....VF..T.S  180

Pantoea - EsaR  181  DDKQQLGGDVVSRTGCPPVAVTTWPLTLPV  210
4Rx13   - EsaR  181  E.QNRMASGI.TGL...RSS.I....S...  210


ALIGNMENTS
>WP_004950750.1 LuxR family transcriptional regulator [Serratia plymuthica]
 AGO53108.1 transcriptional activator protein EsaR [Serratia plymuthica 4Rx13]
Length=249

 Score = 411 bits (1057),  Expect = 2e-148, Method: Compositional matrix adjust.
 Identities = 198/249 (80%), Positives = 218/249 (88%), Gaps = 0/249 (0%)

Pantoea - EsaI  1    MFSFFLENQTITDTLQTYIQRKLSPLGSPDYAYTVVSKKNPSNVLIISSYPDEWIRLYRA  60
4Rx13   - EsaI  1    ...V.NK.....E..RDH.D....S....E......R.....D...........VD....  60

Pantoea - EsaI  61   NNFQLTDPVILTAFKRTSPFAWDENITLMSDLRFTKIFSLSKQYNIVNGFTYVLHDHMNN  120
4Rx13   - EsaI  61   ................................K.............I....F........  120

Pantoea - EsaI  121  LALLSVIIKGNDQTALEQRLAAEQGTMQMLLIDFNEQMYRLAGTEGERAPALNQSADKTI  180
4Rx13   - EsaI  121  ........AN.G.EE..K..SS.R.A.......I.........AASSSNQKYHTD...P.  180

Pantoea - EsaI  181  FSSRENEVLYWASMGKTYAEIAAITGISVSTVKFHIKNVVVKLGVSNARQAIRLGVELDL  240
4Rx13   - EsaI  181  .TP..............G.....A..........MG.I...................E.  240

Pantoea - EsaI  241  IRPAASAAR  249
4Rx13   - EsaI  241  .T...T...  249
```

Figure S4: Alignment of EsaI/R (*Pantoea stewartii* spp. *stewartii*) with EsaI/R (*S. ply.* 4Rx13. Alignment was performed using Protein BLAST analysis (NCBI, Altschul *et al.* 1990). Deviating amino acids are indicated in bold red letters. Matches are represented by dots.

8.7 *Lux*S expression in *S. ply.* 4Rx13 *cya*::Km and *crp*::Km

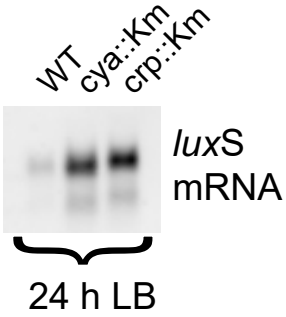

Figure S5: Expression of *lux*S in *S. ply.* 4Rx13 *cya*::Km and *crp*::Km. Expression levels of the *lux*S gene were determined by Northern blot, using 5 µg RNA after 24 h cultivation in complex medium (LB). A DIG-dUTP labelled probe was used. Hybridization was visualized by fluorenscence measurements for 1 min.

Curriculum vitae

Persönliche Daten

Name	Nancy Magnus
Geburtsdatum	21.11.1986
Nationalität	Deutsch
Familienstand	Ledig

Arbeitserfahrung

Aktuelle Stelle

seit 2012 **Doktorand**, *Universität Rostock, Lehrstuhl für Biochemie*.
Dissertationsthema: Unraveling the regulatory mechanisms controlling the biosynthesis and emission of the volatile organic compound 'sodorifen' produced by *Serratia plymuthica* 4Rx13.
Koordination des Austauschprogramms mit der Babeș-Bolyai Universität Klausenburg, Rumänien (seit 2016)

Ausbildung

2006 –2012 **Diplom Biologie**, *Universität Rostock, Lehrstugl für Biochemie*, Rostock, Gesamtnote: 1,1.
Thema der Diplomarbeit: Untersuchungen zum Plasmid von *Serratia odorifera* 4Rx13

Lehrveranstaltungen (Universität Rostock)

SoSe 2017/18	Praktikum	*Biochemisches Grundpraktikum*
SoSe 2015	Tutorium	*Biochemie*

Auszeichnungen und Drittmittel

2013 –2015 **Stipendium der Landesgraduiertenförderung Mecklenburg-Vorpommern**.

2014 **Präsentationspreis auf der MiCom (4th International Student Conference on Microbial communication)**, *Analyses of potential biosynthetic genes of the volatile organic compound ‚sodorifen' emitted by Serratia plymuthica 4Rx13*, Jena.

Mitgliedschaften

- Vereinigung für Allgemeine und Angewandte Mikrobiologie (VAAM)
- Graduiertenakademie der Universität Rostock

Sprachkenntnisse

Deutsch Muttersprache

English	verhandlungssicher, C1-Level
Französisch	Grundkenntnisse

Programmkenntnisse

Sehr gut	Microsoft Office, BLAST, SigmaPlot, Origin
Gut	LaTeX , antiSMASH

Publikationen und Konferenzbeiträge

Artikel mit Peer-Review

[1] Magnus, N., Weise, T., and Piechulla, B. Carbon Catabolite Repression Regulates the Production of the Unique Volatile Sodorifen of Serratia plymuthica 4Rx13. *Frontiers in microbiology*, 8:2522, 2017.

[2] Domik, D., Magnus, N., and Piechulla, B. Analysis of a new cluster of genes involved in the synthesis of the unique volatile organic compound sodorifen of *Serratia plymuthica* 4Rx13. *FEMS microbiology letters*, 363(14), 2016.

Artikel in Begutachtung

[1] Meoded, R. A., Ueoka, R., Helfrich, E. J., Jensen, K., Magnus, N., Piechulla, B., and Piel, J. A polyketide synthase component for oxygen insertion into polyketide backbones.

Konferenzbeiträge

[1] Nancy Magnus. Analyses of potential biosynthetic genes of the volatile organic compound ‚sodorifen' emitted by *Serratia plymuthica* 4Rx13: Vortrag auf der MiCom - 4th International Student Conference on Microbial Communication, 03.04.2014, Jena.

[2] Nancy Magnus, Dajana Domik, Teresa Weise, and Birgit Piechulla. Analysing the regulatory mechanisms underlying the 'sodorifen' emission in *Serratia plymuthica* 4Rx13: Poster präsentiert auf der Jahrestagung 2014 der Vereinigung für Allgemeine und Angewandte Mikrobiologie, 7.10.2014, Dresden.

[3] Nancy Magnus and Birgit Piechulla. The role of carbon catabolite repression in the emission of ‚sodorifen' by *Serratia plymuthica* 4Rx13: Poster präsentiert auf der Jahrestagung 2016 der Vereinigung für Allgemeine und Angewandte Mikrobiologie, 15.03.2016, Jena.

[4] Nancy Magnus, Elisa Teichmann, Eric Zitzow, Thoralf Bernhardt, and Birgit Piechulla. The biosynthesis of the unique volatile sodorifen is regulated on multiple levels: Poster präsentiert auf der Jahrestagung 2017 der Vereinigung für Allgemeine und Angewandte Mikrobiologie, 07.03.2017, Würzburg.

Rostock, 21.03.2018

Danksagung

Als erstes möchte ich mich bei meiner Betreuerin Birgit Piechulla für die Möglichkeit bedanken, dieses interessante Thema zu bearbeiten. Außerdem für die Möglichkeiten meine Ergebnisse auf Tagungen zu präsentieren und für die Begutachtung dieser Arbeit.

Ebenfalls bedanken möchte ich mich bei dem zweiten Gutachter für das Lesen und Beurteilen meiner Arbeit.

Ein besonderes Dankeschön geht an Teresa Weise. Du hast mich während meiner Diplomarbeit und dem Beginn der Dissertation betreut und ich konnte so so so viel von dir lernen, was ich nun an die nachfolgenden Generationen an Bachelor- und Masterstudenten weitergeben kann. Du warst ein tolles Vorbild!

Riesengroßer Dank geht natürlich auch an alle meine Kollegen, Chantal, Claudi, Dörte, Kerstin, Marco, Steffi und Uta, die das Arbeiten so angenehm gemacht haben. Chantal und Uta, ihr wart eine große Stütze in den letzten Jahren und habt mir durch so manche Krise geholfen und danke auch für das Korrekturlesen ;). Bei Claudi und Steffi möchte ich mich für die Hilfe bei den Unmengen an VOC-Systemen und Northern Blots bedanken! Ohne euch, wäre ich jetzt wahrscheinlich noch nicht fertig ;) Dörte, du bist/warst für mich eine ganz besondere Kollegin, zu der ich immer gehen konnte, wenn es irgendwo hakte, sei es beruflich oder privat. Danke dafür! Bedanken möchte ich mich auch bei allen Studenten, die ich im Laufe meiner Dissertation ein Stück ihres Weges begleiten durfte.

Ein großer Dank geht ebenfalls an mein "'Wochenend-Kind"' Maria, die stets für den nötigen Ausgleich zur Arbeit gesorgt hat. Auf das wir noch lange zusammen durch die Welt "tottern". Danke auch an meine Patenkinder Akira, Nala und Chanel. Ihr habt mich immer wieder vor ungeahnte Herausforderungen gestellt, die ich mit der Zeit gelernt habe zu meistern! Ich hab euch lieb!

Ganz besonders möchte ich mich natürlich auch bei meinen anderen Freunden für die Unterstützung in dieser, oftmals turbulenten, Zeit bedanken! Besonders bei meiner allerliebsten Dine! Wir kennen uns seit der Schulzeit und haben uns trotz einiger Tiefen nie aus den Augen verloren. Ich bin so froh dich zu haben!

Ich bin ebenfalls für die Unterstützung meiner Familie sehr dankbar. Tom Schmidt, danke dass du immer für mich da bist, wenn ich dich brauche. Ebenso dankbar bin ich für die Unterstützung meiner Großeltern Marie-Luise und Karl-Heinz Schmidt, die nie daran gezweifelt haben, dass dieser Tag kommt. Danke auch an meine gesamte "'Schwieger-Familie"' dafür dass ihr mich so herzlich in eurer Mitte aufgenommen habt.

Mein herzlichstes Dankeschön geht an meine Mama, Beate Magnus. Du hast immer an mich geglaubt und mich stets in allen meinen Entscheidungen unterstützt. Ohne dich hätte ich diesen Punkt wahrscheinlich nie erreicht! Du bist die Beste und ich hab dich lieb!

Natürlich dürfen bei all den Dankesbekundungen meine beiden Tierkinder Pinsel und Flocke nicht fehlen! Ohne euch wäre das Leben nur halb so lustig. Vor allem meine kleine Möchtegern-Mexikanerin Flocke hat mir so manchen harten Arbeitstag versüßt und mir immer wieder ein Lächeln ins Gesicht gezaubert!

Last but not least, ein riesen-, riesengroßer Dank geht an dich, Daniel. Seit fast schon 9 Jahren hälst du es mit mir aus und hast öfter mal meinen Frust ungerechterweise abbekommen. Entschuldige! Mindestens genauso oft hast du mich aber auch wieder aufgebaut, wenn mal wieder nichts klappte und hast immer an mich geglaubt. Herzlichen Glückwunsch. Du kannst dich nun, verdienterweise, "'Herr der Frau Doktor"' nennen :oP Ich liebe dich!