Regulation and natural functions of lipopeptide biosynthesis in *Pseudomonas*

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Regulation and natural functions of lipopeptide biosynthesis in *Pseudomonas*

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Thesis

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Chapter 1

General Introduction

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Introduction

The genus *Pseudomonas* is well-known for its diverse life-styles, its distribution in a wide range of environments and its production of an array of secondary metabolites. The genus harbours both pathogenic and beneficial species with *P. aeruginosa, P. entomophila* and *P. syringae* as the best studied pathogenic species of animals, insects and plants, respectively. Next to these pathogenic species, the genus *Pseudomonas* harbours multiple species that can have diverse beneficial effects in soil, water or on/in tissues of eukaryotes. For example, *P. putida* is of substantial interest for its bioremediation properties that allow degradation or detoxification of hazardous environmental contaminants (Loh & Cao, 2008). *P. fluorescens* harbours many strains that affect plant growth and induce systemic resistance in plants against plant pathogenic microorganisms (Haas & Defago, 2005). *P. fluorescens* is well represented in the plant rhizosphere but is also found in diverse other habitats, including phyllosphere, soil and water. The genus *Pseudomonas* currently comprises more than 100 named species that have been divided into lineages, groups and subgroups based on multilocus sequence analysis (Yamamoto *et al.*, 2000, Mulet *et al.*, 2010). Many of the plant commensal strains fall into the *Pseudomonas fluorescens* group, which currently includes more than fifty named species (Mulet et al., 2010). The *P. fluorescens* group includes *P. chlororaphis*, *P. protegens*, *P. brassicacearum*, *P. fluorescens* and *Pseudomonas* sp..

Irrespective of their different life styles, *Pseudomonas* species typically produce a range of secondary metabolites that allow them to invade and infect host tissue, to degrade xenobiotic compounds, to promote plant growth or to protect plants from pathogen infection. Among the secondary metabolites produced by *Pseudomonas* species, the lipopeptides (LPs) stand out for their amphiphilic properties, their antimicrobial activities and their role in swarming and biofilm formation (Raaijmakers *et al.*, 2010). The **overall aim of the research described in this thesis** was to elucidate the regulatory pathways of LP biosynthesis and to unravel the natural functions of LPs produced by different *Pseudomonas* species. In this introductory chapter, I will first give a brief overview of *Pseudomonas* comparative genomics studies with emphasis on gene and gene clusters involved in the production of secondary metabolites. I will then summarize the current knowledge of LP biosynthesis and regulation and highlight the natural functions of LPs produced by *Pseudomonas* and other bacterial genera. Finally I will outline the scope of my PhD thesis.

Pseudomonas genomes and secondary metabolism

The taxonomy of the genus *Pseudomonas* has been largely determined by distinct phenotypic features, biochemical properties, DNA-DNA hybridization, and sequences of the ribosomal RNA (rRNA) and specific housekeeping genes (Moore *et al.*, 1996, Maiden *et al.*, 1998, Gardan *et al.*, 1999, Yamamoto et al., 2000, Goris *et al.*, 2007). The first available complete genome sequence of a *Pseudomonas* species was that of *P. aeruginosa* strain PAO1 which was published by Stover et al (2000). In the years thereafter, several research groups generated genome sequences of their own favourite *Pseudomonas* strain(s). In our research group in Wageningen, the Netherlands, we focused primarily on *P. fluorescens* strain SS101. At this moment (January 2015), 73 complete *Pseudomonas* genomes are listed in NCBI's database, with another 630 listed as being draft assemblies or incomplete. With this increasing amount of genome sequences, it is now possible to conduct more in-depth analyses of the genomic and genetic characteristics of the *Pseudomonas* genus and to better define their lineages. Silby et al. (2011) compared genome data of several *Pseudomonas* spp. and genome sizes vary substantially, ranging from 4.5 Mb for *P. stutzeri* to 7.0 Mb for *P. protegens* Pf-5. They further demonstrated that *Pseudomonas* genomes have a relatively high GC content ranging from 57.9% to 66.6%. To show close relationships among different strains of *P. aeruginosa*, they combined both DNA hybridization and genome-based analyses. For the *P. fluorescens* group, however, Silby et al. (2011) recognized the large genomic diversity and proposed that this group is a species complex.

In 2012, a research consortium comprising 15 research groups including ours published a comparative genomics study of 10 plant-associated *Pseudomonas* spp. all belonging to the *P. fluorescens* group (Loper *et al.*, 2012). This study, which included 3 previously sequenced strains and 7 newly sequenced strains, substantially extended the number of genome sequences of the *P. fluorescens* group and defined three sub-clades based on multilocus sequence analysis (Figure 1). Consequently, strain Pf-5 is now classified as *P. protegens* and falls into sub-clade 1 together with two *P. chlororaphis* strains. Sub-clade 2 is composed of the closely related *P. fluorescens* Q2-87 and *P. brassicacearum* Q8r1-96 and the previously-sequenced strain *P. fluorescens* Pf0-1. Of the four members in sub-clade 3, *P. fluorescens* A506 and *P. fluorescens* SS101 are the most closely related, this clade also includes the previously-sequenced *P. fluorescens* SBW25 and *Pseudomonas* sp. BG33R. Moreover, our comparative genomic study revealed an enormous heterogeneity among the genomes of strains in the *P. fluorescens* group, with a pan genome of 13872 predicted genes and a core genome of only 2789 genes, representing 45%-52% of the genome of each of the ten strains included in the analysis (Figure 2). This genomic heterogeneity reflects, to some extent, the specific lifestyles and plasticity of these strains and provides genomic insight into their ecological, physiological and metabolic diversi

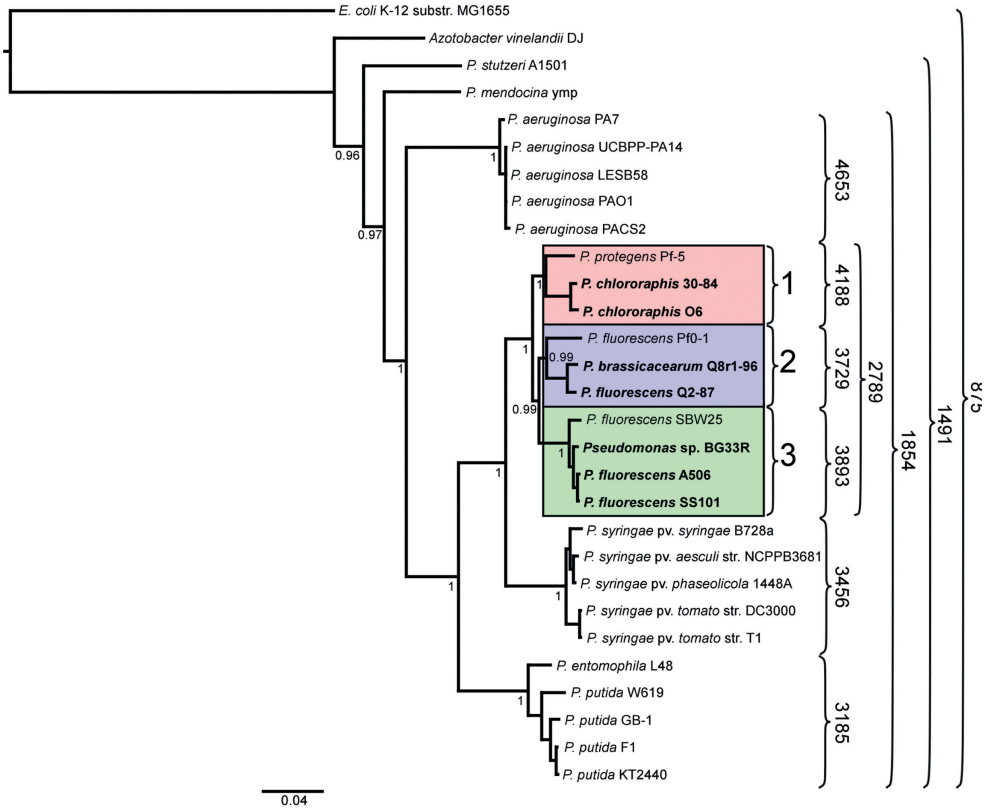

Figure 1. Phylogenetic tree depicting the relationships of sequenced strains of *Pseudomonas* spp. The tree is based on concatenated alignments of ten core housekeeping genes: *acsA*, *aroE*, *dnaE*, *guaA*, *gyrB*, *mutL*, *ppsA*, *pyrC*, *recA*, and *rpoB*, and was generated using the MrBayes package (Ronquist & Huelsenbeck, 2003). The interior node values of the tree are clade credibility values, which represent the likelihood of the clade existing, based on the posterior probability values produced by MrBayes. Strains in the *P. fluorescens* group fall within a single clade comprised of three sub-clades, which are numbered 1 to 3 and highlighted pink, blue and green, respectively. Strains sequenced in the study are in bold font. Numbers on the right of the figure represent the size of the core genome of the strains included within the curved brackets. This figure is reproduced from (Loper et al., 2012).

The genus *Pseudomonas* produces an array of secondary metabolites, including enzymes, volatiles, bacteriocins, toxins, antibiotics, and LPs (Raaijmakers et al., 2010, Raaijmakers *et al.*, 2006, Haas & Defago, 2005). Many of these metabolites have been investigated for their antimicrobial activities, in particular 2,4-diacetylphloroglucinol (Raaijmakers *et al.*, 1997), pyoluteorin (Bender *et al.*, 1998), pyrrolnitrin (Howell & Stipanovic, 1979), hydrogen cyanide (Voisard *et al.*, 1989), syringomycin (Sorensen *et al.*, 1996), syringopeptin (Lavermicocca *et al.*, 1997), viscosinamide (Nielsen *et al.*, 1999), viscosin (Neu *et al.*, 1990, de Bruijn *et al.*, 2007), thioquinolobactin (Matthijs *et al.*, 2007), phenazines (Thomashow & Weller, 1988) and other yet to be identified compounds (Garbeva & de Boer, 2009). Our analyses of the ten *Pseudomonas* genomes

showed that the majority of the secondary metabolite gene clusters has a patchy distribution, indicating a complex pattern of inheritance including several independent acquisition events and/or loss of the clusters from the genomes of certain strains. The genome analyses also revealed candidate genes and gene clusters that encode putative novel metabolites with yet unknown functions. These metabolites might be needed to support the life style of these bacteria or play a role in interactions with plants, other microbes and insects. For instance, biosynthesis genes were found for i) hemophores , ii) novel bacteriocins, iii) type II, III, VI secretion systems (T2SS, T3SS and T6SS, respectively), and iv) novel insecticidal toxins (Figure 3). The predicted hemophore may have a function in chelating heme and then be bound and taken up by specific outer membrane receptors. Bacteriocins are narrow-spectrum proteinaceous toxins that can kill bacteria of closely related strains of a given species. Each of the ten genomes of the *P. fluorescens* group has two to seven genes or gene clusters encoding predicted bacteriocins (Figure 3), of which are three putative novel bacteriocins. The first class is designated as N1 and appears in all strains except Pf-5. The second class of putative novel bacteriocins is N2, which is found in four strains. The third class is N3, present only in the BG33R genome. Many of the bacteriocin genes are located in genomic islands or other atypical genomic regions, suggesting that these genes are the result of horizontal transfer events.

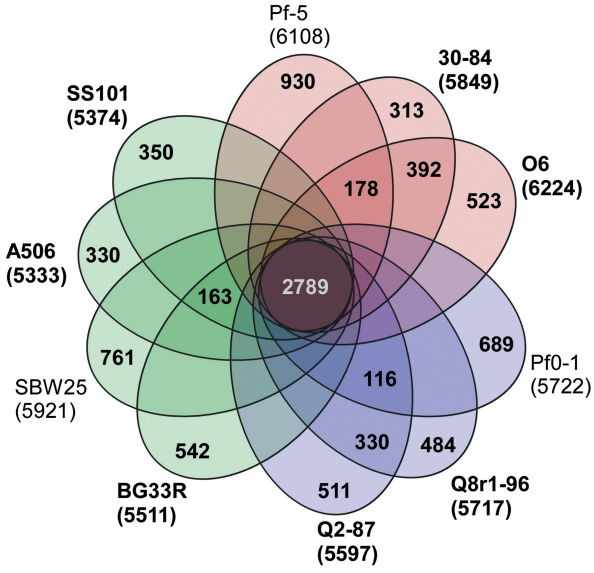

Figure 2. Genomic diversity of strains in the *P. fluorescens* group. Each strain is represented by an oval that is colored according to sub-clade (as in Figure 1). The number of orthologous coding sequences (CDSs) shared by all strains (i.e., the core genome) is in the center. Overlapping regions show the number of CDSs conserved only within the specified genomes. Numbers in non-overlapping portions of each oval show the number of CDSs unique to each strain. The total number of protein coding genes within each genome is listed below the strain name. Strains sequenced in the study are in bold font. This figure is reproduced from (Loper et al., 2012).

Many extracellular enzymes are transported out of the cell through T2SS. Four T2SSs were found in these ten genomes, three of which are related to the Xcp and Hxc systems of *P. aeruginosa*. The fourth T2SS is a novel one and found only in species belonging to sub-clade 3. T3SSs and T6SSs function in delivery of effector molecules into plant, animal or bacterial cells. Several of these secretion systems were identified in the *P.*

fluorescens group. With respect to the insecticidal toxins, the genomes contain six distinct types of Tc (toxin complexes) gene clusters and only sub-clade 1 possesses the *fit* (fluorescens insect toxin) gene cluster.

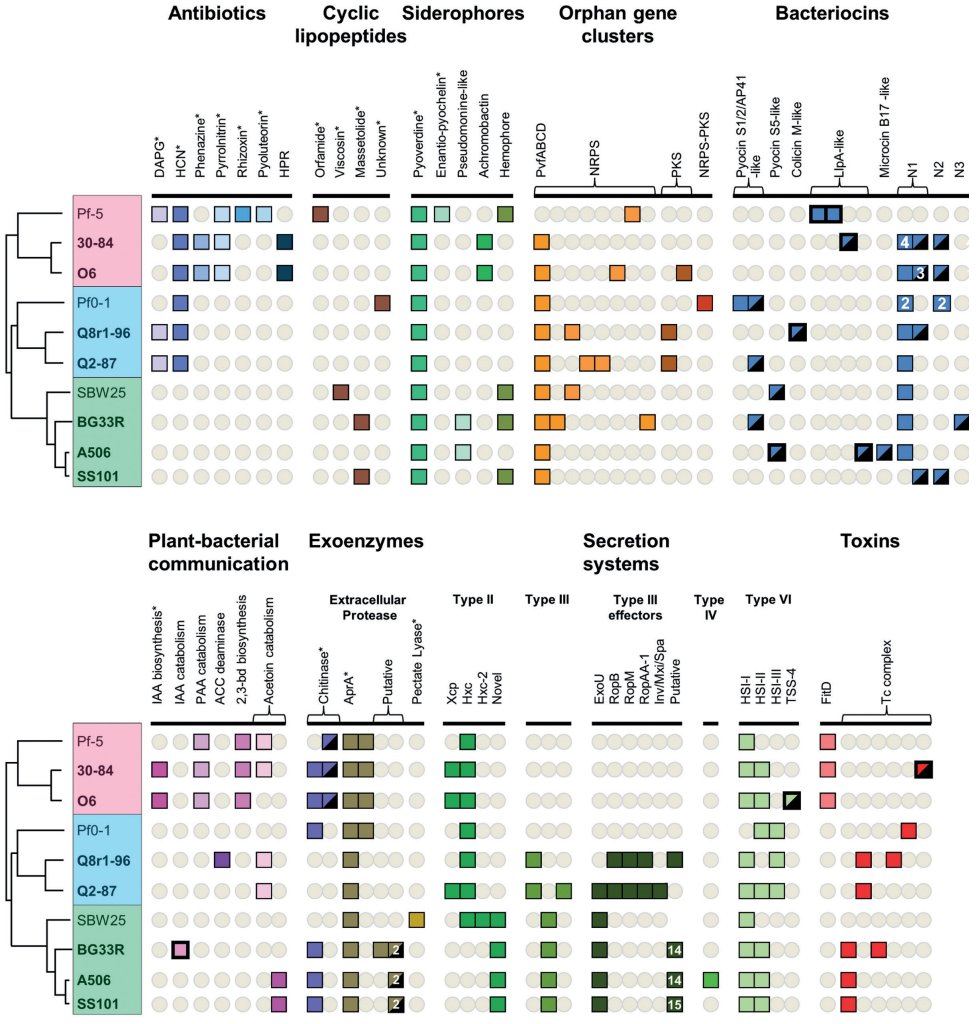

Figure 3. Selected biosynthetic/catabolic genes or gene clusters in the sequenced strains of the *P. fluorescens* group. Colored boxes represent the presence of a gene or gene cluster within a genome, while absence of a cluster is represented by a grey circle; numbers within a box represent the number of copies of a gene or cluster within a genome. Putative T3SS effectors were not examined for SBW25, therefore no box or circle is present in that column for SBW25. Genes within a mobile genetic element have the box outline bolded; genes within regions of atypical trinucleotide content have half of their boxes blackened. Abbreviations are as follows: 2,4-diacetylphloroglucinol (DAPG); hydrogen cyanide (HCN); derivatives of rhizoxin (Rhizoxins); 2-hexyl-5-propyl-alkylresorcinol (HPR); nonribosomal peptide synthetase (NRPS); polyketide synthase (PKS); novel groups 1–3, respectively, of the carocin- and pyocin-like bacteriocins found in these strains (N1, N2, N3); indole-3-acetic acid (IAA); phenylacetic acid (PAA); aminocyclopropane-1-carboxylic acid (ACC); type VI secretion systems found within virulence loci HSI-I, HSI-II, and HSI-III, respectively, of *P. aeruginosa* (HSI-I, II, II); TSS-4 from *Burkholderia* pseudomallei (TSS-4). Asterisks indicate that the expected phenotype is known to be expressed or was detected in this study by the strains having the indicated genes or gene clusters. This figure is reproduced from (Loper et al., 2012).

For the LP biosynthesis genes, the comparative genome analyses showed that genes for orfamide A biosynthesis are found in a single cluster in the *P. protegens* Pf-5 genome, whereas genes for the biosynthesis of massetolide A and viscosin are present at two distinct locations in the genomes of *P. fluorescens* SS101 and SBW25, respectively. Moreover, we identified gene clusters for LP biosynthesis in the genomes of BG33R and Pf0-1. Although the structures of the LPs are yet unknown, the amino acid composition of their peptide moieties was predicted from specific signature sequences in the adenylation domains of the encoded proteins. The predicted structure of the LP in BG33R is similar to that of massetolide or pseudophomin A and B. The LP produced by Pf0-1 was predicted to consist of an 11 amino acid peptide moiety. Collectively, the comparative genome analyses of multiple *Pseudomonas* species and strains provided new insights into their metabolic diversity and the presence of new and unique genes involved in the biosynthesis of yet unknown bioactive metabolites.

Lipopeptide biosynthesis in *Pseudomonas*

LPs are composed of a lipid tail linked to a (cyclic) oligopeptide (Figure 4). They are synthesized by large nonribosomal peptide synthetases (NRPSs) via a thiotemplate process. For a more detailed understanding of the structural and functional characteristics of the enzymes involved, we refer to the reviews by Raaijmakers et al. (2006) and Ongena & Jacques (2008). Based on the length, composition of the fatty acid tail, and the number, type and configuration of the amino acids in the peptide moiety, *Pseudomonas* LPs were initially classified into four main groups: the viscosin, amphisin, tolaasin and syringomycin (Raaijmakers et al., 2006). The viscosin group harbours LPs with 9 amino acids linked at the N-terminus to, in most cases, 3-hydroxydecanoic acid (3-HDA). This group includes viscosin, massetolide A, pseudophomins, pseudodesmins, viscosinamide, and White-Line-Inducing Principle (WLIP) (Table 1) (de Bruijn et al., 2007, de Bruijn *et al.*, 2008, Quail *et al.*, 2002, Pedras *et al.*, 2003, Nielsen et al., 1999). The biosynthesis gene clusters of viscosin and massetolide A have been described in detail (de Bruijn et al., 2007, de Bruijn et al., 2008). The amphisin group, consisting of amphisin, arthrofactin, lokisin, pholipeptin and tensin, represents LPs with peptides of 11 amino acids coupled to 3-HDA (Koch *et al.*, 2002, Roongsawang *et al.*, 2003, Sorensen *et al.*, 2002, Ui *et al.*, 1997, Nielsen *et al.*, 2000). So far, the only well-characterized biosynthetic gene cluster from this group is arthrofactin, whose biosynthesis is governed by three NRPS genes designated *arfA*, *arfB* and *arfC* (Roongsawang et al., 2003). The tolaasin group, with the LPs tolaasin, syringopeptin, corpeptin and sesillin, is more diverse in terms of the composition and length of the peptide chain of the LPs (19-25 amino acids) as well as the lipid tail (3-HDA or 3-hydroxyoctanoic acid (3-HOA)). The biosynthesis gene cluster of syringopeptin contains three large open reading frames *sypA*, *sypB*, and *sypC*, that are 16.1, 16.3, and 40.6 kb in size, respectively (Scholz-Schroeder *et al.*, 2003). Similar to the viscosin group of LPs, also the syringomycin

group contains LPs composed of 9 amino acids. However, LPs in the syringomycin group distinguish themselves from viscosin-like LPs through the occurrence of unusual amino acids such as didehydroaminobutyric acid (Dhb), 2,4-diamino butyric acid (Dab), and the C-terminal chlorinated threonine residue.

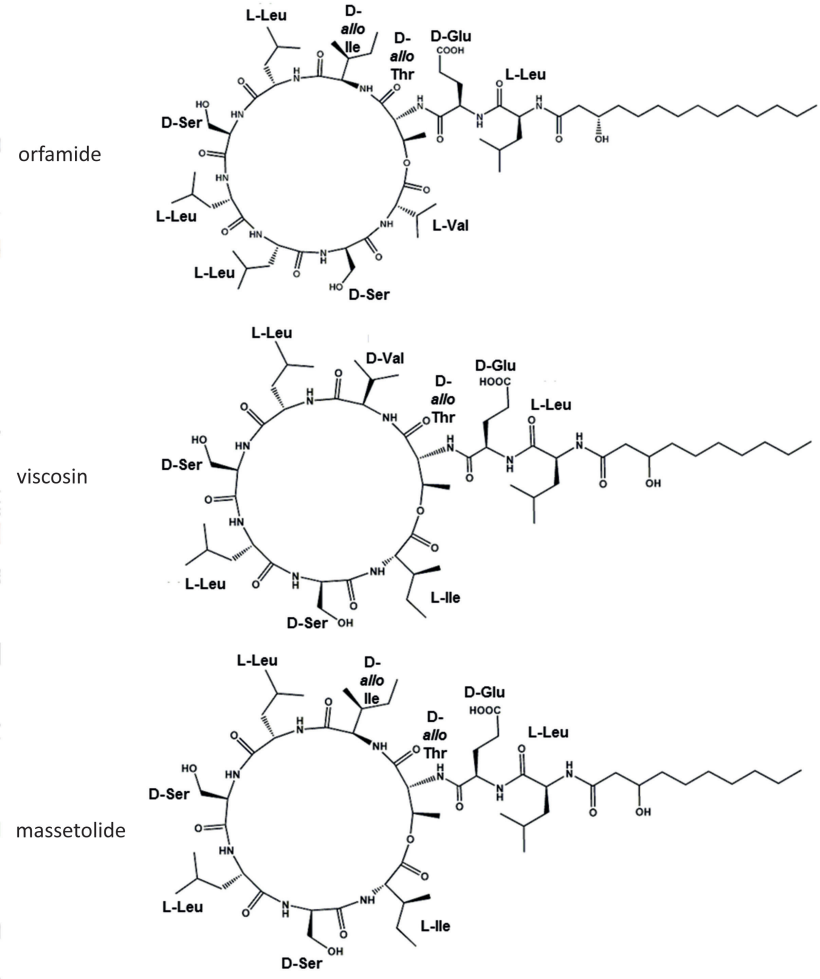

Figure 4. Structures of lipopeptides (LPs) production by the three strains in the *P. fluorescens* group studied in this PhD research. Orfamide, viscosin and massetolide are produced by *P. protegens* Pf-5, *P. fluorescens* SBW25 and *P. fluorescens* SS101 respectively.

With the increasing availability of whole genome sequences of *Pseudomonas* over the past decade, more and different LP biosynthesis genes were discovered, leading to a substantial extension of the chemical classification initially proposed by Raaijmakers et al. (2006). Genome analysis of *P. protegens* Pf-5 followed by a genome-isotopic approach led to the identification of the orfamides, LPs with 10 amino acids and

a 3-hydroxy myristic acid (3-HMA) tail. (Paulsen *et al.*, 2005, Gross *et al.*, 2007). Subsequently, structural orfamide analogues were identified in *Pseudomonas* CMR12a, a strain isolated from the rhizosphere of cocoyam (D'Aes *et al.*, 2014). This strain also produces sessilin, a LP that differs in only one amino acid from tolaasin, the toxin produced by the mushroom pathogen *P. tolaasii* (D'Aes et al., 2014). Following similar approaches Dubern et al (2008) identified the 12-amino acid LPs putisolvins I and II and their corresponding gene cluster in *P. putida* PCL1445. In another *P. putida* strain RW10S2, the WLIP biosynthesis genes *wlpA*, *wlpB*, and *wlpC* were identified. They are distributed over two separate gene clusters, an organization similar to that of the viscosin and massetolide biosynthesis genes. In the plant pathogen *P. cichorii* strain SF1-54, cichofactin A and B were discovered, linear LPs with decanoic and dedecanoic lipid chains connected to the N-terminus of an 8-amino acid peptide moiety (Pauwelyn *et al.*, 2013). The proteins encoded by biosynthesis genes *cifA* and *cifB* have 76% and 75% identity to those encoded by *syfA* and *syfB*, respectively, two syringafactin biosynthesis genes present in *P. syringae* pv *tomato* DC3000. Also the genetic backbone of the xantholysin NRPS system in *P. putida* BW11M1 bears considerable similarity to the one for entolysin biosynthesis of *P. entomophila* L48, both consisting of a fourteen-unit assembly line with three enzymes operating in co-linear mode. Despite this overall similarity, the peptide sequences of xantholysin and entolysin differ in at least six positions (Li *et al.*, 2013, Vallet-Gely *et al.*, 2010). Last but not least was the discovery of LPs consisting of just 2 amino acids (Thirkettle *et al.*, 2000, Busby *et al.*, 2000, Andersson *et al.*, 2012, Schmidt *et al.*, 2014). SB-253514 is a bicyclic carbamate first isolated from *P. fluorescens* DSM 11579 (Busby et al., 2000, Thirkettle et al., 2000), and subsequently from *Pseudomonas brassicacearum (Andersson et al., 2012)*. More recently, structurally similar compounds were isolated from the plant-associated *Pseudomonas* sp. SH-C52 and designated as brabantamides. Bioinformatic analysis showed that the *braB* gene in SH-C52 encodes a bimodular NRPS that recognizes serine and proline. The NRPS assembly line first leads to production of a linear di-lipopeptide, then the linear di-lipopeptide is cyclised into a 5,6-bicyclic intermediate, and finally, this is interconverted into the glycosylated brabantamides (Schmidt et al., 2014).

Collectively, these and other genome- and chemical-based discoveries of structurally new LPs confirm and extend the enormous flexibility and versatility of nonribosomal biosynthesis of secondary metabolites. These findings also show that the initial classification into four main groups needs to be revisited.

Table 1. Genes involved in the biosynthesis and regulation of Lipopeptides (LPs) by *Pseudomonas* strains

LP group	LP	Species/strains	Gene/protein information	References	Regulation genes	References
viscosin	viscosin	*P. fluorescens* PfA7B	nonribosomal peptide synthetases	Braun et al., 2001	-	
	viscosin	*P. fluorescens* 5064	-		AHL biosynthesis	Cui et al., 2005
	viscosin	*P. fluorescens* SBW25	*viscA*, *viscB*, *viscC*	de Bruijn et al., 2007	GacA/GacS two-component system	de Bruijn et al., 2007
					luxR transcriptional regulator	de Bruijn & Raaijmakers, 2009a
	massetolide A	*P. fluorescens* SS101	*massA*, *massB*, *massC*	de Bruijn et al., 2008	GacA/GacS two-component system; *luxR* transcriptional regulator	de Bruijn & Raaijmakers, 2009a
					clpP serine protease	de Bruijn & Raaijmakers, 2009b
	massetolide A	*P. fluorescens* BG33R	*massA*, *massB*, *massC*	Loper et al., 2012	-	
	white-line-inducing principle (WLIP)	*P. putida* RW10S2	*wlpA*, *wlpB*, *wlpC*	Rokni-Zadeh et al., 2012	*gacS* sensor kinase	Rokni-Zadeh et al., 2012
	white-line-inducing principle (WLIP)	*P. reactans* LMG 5329	*wipA*, *wipB*, *wipC*	Rokni-Zadeh et al., 2013	*luxR* transcriptional regulator *wlpR*	Rokni-Zadeh et al., 2013
	white-line-inducing principle (WLIP)	*P. fluorescens* BRG100	-	Quail et al., 2002, Pedras et al., 2003	-	
	pseudodesmins A and B	*Pseudomonas*	-	Sinnaeve et al., 2009	-	
	viscosinamide	*P. fluorescens* DR54	-	Nielsen et al., 1999	-	

LP group	LP	Species/strains	Gene/protein information	References	Regulation genes	References
amphisin	amphisin	*Pseudomonas* sp. strain DSS73	*amsY*, peptide synthetase	Koch et al., 2002	GacA/GacS two-component system	Koch et al., 2002
	arthrofactin	*Pseudomonas* sp. MIS38	*arfA*, *arfB*, *arfC*	Roongsawang et al., 2003	ORF1; putative DNA binding protein (luxR type)	Roongsawang et al., 2003
					Multiple ATP dependent active transporter systems are responsiblefor the production	Lim et al., 2009
					SyrF-like protein (*arfF*), heat shock protein (*htpG*), and (p)ppGpp synthetase/hydrolase (*spoT*)	Washio et al., 2010, Washio et al., 2011
	lokisin	*Pseudomonas* sp. strain DSS41	-	Sorensen et al., 2002	-	
	pholipeptin	*P. fluorescens*	-	Ui et al., 1997	-	
	tensin	*P. fluorescens* strain 96.578	-	Nielsen et al., 2000	-	
tolaasin	tolaasin	*P. tolaasin*	TL1, TL2, TL3 high molecular weight protein	Rainey et al., 1993, Bassarello et al., 2004	PheN, two component system regulatory protein (*gacS*)	Grewal et al., 1995
	tolaasin	*Pseudomonas* NZ17	-	Godfrey et al., 2001	-	
	syringopeptin	*P. syringae*.pv.*syringae* B728a	syringopeptin sythetase genes	Feil et al., 2005	*gidA*, initiation of chromosome replication	Kinscherf & Willis, 2002
	syringopeptin	*P. syringae*.pv.*syringae* B301D	*sypA*, *sypB*, *sypC*	Scholz-Schroeder et al., 2003, Scholz-Schroeder et al., 2001	*salA*, *syrG*, *syrF,* putative DNA-binding regulatory proteins (luxR type);	Lu et al., 2002
					Plant signal molecules	Wang et al., 2006
					pseF, an ABC-type cytoplasmic membrane protein	Cho & Kang, 2012
	corpeptin	*P. corrugata*	-	Emanuele et al., 1998	PcoI/PcoR quorum sensing system	Licciardello et al., 2012
	sesillin	*Pseudomonas* sp.CMR12a	*sesA, sesB*, *sesC*	D'Aes et al., 2014	LuxR type regulator	D'Aes et al., 2014

LP group	LP	Species/strains	Gene/protein information	References	Regulation genes	References
syringomycin	syringomycin	*P. syringae*.pv.*syringae* B728a	syringomycin synthetase genes	Feil et al., 2005	*gidA*, initiation of chromosome replication	Kinscherf & Willis, 2002
					lemA, two component system regulatory protein (*gacS*)	Hrabak & Willis, 1992, Kitten et al., 1998
					gacA response regulator	Rich et al., 1994, Feil et al., 2005
					salA putative DNA-binding protein (luxR type)	Kitten et al., 1998
	syringomycin	*P. syringae*.pv.*syringae* B301D	*syrE*	Guenzi et al., 1998, Scholz-Schroeder et al., 2001	*salA*, *syrG*, *syrF* putative DNA-binding proteins (*luxR* type);	Lu et al., 2002, Wang et al., 2006
			syrB1, *syrC*	Zhang et al., 1995, Guenzi et al., 1998	*syrA*, N-acetylglutamate synthase (arginine biosynthesis)	Lu et al., 2003
			syrB2	Vaillancourt et al., 2005	*syrP*, histidine kinase in two-component regulatory system (phosphorelay)	Zhang et al., 1997
					pseF, an ABC-type cytoplasmic membrane protein	Cho & Kang, 2012
	cormycin	*P. corrugata*	-	Scaloni et al., 2004	PcoI/PcoR quorum sensing system	Licciardello et al., 2012
	syringostatin	*P. syringae* pv. *syringae*	-	Sorensen et al., 1996	-	
	syringotoxin	*P. syringae* pv. *syringae*	-	Sorensen et al., 1996	-	
	pseudomycin	*P. syringae*		Ballio et al., 1994	-	

LP group	LP	Species/strains	Gene/protein information	References	Regulation genes	References
others	putisolvin	*P. putida* PCL1445	*psoA*, *psoB*, *psoC*	Kuiper et al., 2004, Dubern et al., 2008	GacA/GacS two-component system	Dubern et al., 2008
					DnaK, DnaJ, GrpE heat shock proteins	Dubern et al., 2008
					ppuI-rsaL-ppuR quorum sensing system with N-(3-oxo-)decanoyl-L-HSL signal	Dubern et al., 2006
	putisolvin-like	*P. putida* 267	-	Kruijt et al., 2009	-	
	entolysin	*P. entomophila* L48	*etlA*, *etlB*, *etlC*	Vallet-Gely et al., 2010	*etlR, macAB; GacA/GacS two-component system, small RNAs rsmY, rsmZ and repressor proteins rsmA1, rsmA2*	Vallet-Gely et al., 2010
	orfamide	*P. protegens* Pf-5	*orfA*, *orfB*, *orfC*	Paulsen et al., 2005, Gross et al., 2007	GacA/GacS two-component system	Hassan et al., 2010
	orfamide	*Pseudomonas* sp.CMR12a	*orfA*, *orfB*, *orfC*	D'Aes et al., 2014	-	
	unknown CLPs	*P. fluorescens* Pf0-1	Pfl01_2211, Pfl01_2212, Pfl01_2213	Garbeva & de Boer, 2009, Loper et al., 2012	-	
	pseudofactin I and II	*P. fluorescens* BD5	-	Janek et al., 2013	-	
	cichofactin A and B	*P. cichorii* SF1-54	*cifA*, *cifB*	Pauwelyn et al., 2013	-	
	xantholysin	*P. putida* BW11M1		Li et al., 2013	*luxR*-family regulator	Li et al., 2013
	brabantamide A	*Pseudomonas* sp. SH-C52	*braB*	Schmidt et al., 2014	-	

Regulation of lipopeptide biosynthesis in *Pseudomonas*

In contrast to the exciting discoveries of new LP biosynthesis genes in *Pseudomonas* and other bacterial genera, there still is considerable lack of knowledge of the genetic mechanisms underlying the regulation of LP biosynthesis genes and the environmental trigger(s) that induce LP biosynthesis. What we know so far is that the GacA/GacS two-component system functions as a master switch of LP biosynthesis: a mutation in either one of the two genes results in loss of LP production in all *Pseudomonas* species and strains in which this two-component system has been examined so far (Kitten *et al.*, 1998, Koch et al., 2002, Dubern *et al.*, 2006, Hassan *et al.*, 2010, Vallet-Gely et al., 2010, de Bruijn & Raaijmakers, 2009b, Rokni-Zadeh *et al.*, 2012) Recently, Vallet-Gely et al. (2010) showed that two small RNAs (RsmY, RsmZ) are involved in the regulation of entolysin biosynthesis in *P. entomophila*. If and how small RNAs regulate LP biosynthesis in other *Pseudomonas* species is as yet unknown. Also quorum sensing (QS) might play a role in the regulation of LP biosynthesis in some *Pseudomonas* species and strains. For *P. fluorescens* 5064 and *P. putida* PCL1445, for example, *N*-acyl homoserine lactones (N-AHLs) that accumulate when cell density increases were shown to regulate viscosin and putisolvin biosynthesis, respectively (Cui *et al.*, 2005, Dubern *et al.*, 2006). However, for various other strains belonging to the same species, including *P. fluorescens* strains SS101 and SBW25, no evidence was found for a role of *N*-AHL-mediated regulation of LP biosynthesis (Dumenyo *et al.*, 1998, Kinscherf & Willis, 1999, Andersen *et al.*, 2003, de Bruijn *et al.*, 2007, de Bruijn *et al.*, 2008). This suggests that cell-density dependent regulation of LP biosynthesis differs among species and among strains of the same species.

Next to these two global regulatory systems based on GacS/GacA and quorum sensing, pathway-specific LuxR-type transcriptional regulators have been shown to regulate syringomycin, syringopeptin, syringofactin, putisolvin, entolysin, viscosin, massetolide, arthrofaction, WLIP, xantholysin and sessilin biosynthesis (Lu *et al.*, 2002, Wang *et al.*, 2006, Berti *et al.*, 2007, Dubern et al., 2008, de Bruijn & Raaijmakers, 2009a, Vallet-Gely et al., 2010, Rokni-Zadeh et al., 2012, Li et al., 2013, D'Aes et al., 2014, Washio *et al.*, 2010). These LuxR-type regulators do not possess the binding domain characteristic for the QS-type LuxR-regulators, but represent a different LuxR-type regulator family (Wang et al., 2006, de Bruijn & Raaijmakers, 2009a).

In *P. putida*, Dubern et al (2005) identified the Hsp70 heat shock protein encoding gene *dnaK* and its flanking genes *dnaJ* and *grpE* as regulators of putisolvin biosynthesis. They further postulated that DnaK, DnaJ and GrpE may be required for proper folding or activity of other regulators of the putisolvin biosynthesis gene *psoA* or alternatively, for proper assembly of the putisolvin NRPSs (Dubern et al., 2005). In *Pseudomonas* sp. MIS38, another heat shock protein named HtpG was found to be essential in LP arthrofactin biosynthesis. Although the mechanism underlying of HtpG regulated

arthrofactin biosynthesis is not yet known, also here a role of HtpG in proper assembly of the multimodular enzymes was proposed based on earlier work on the role of HtpG in the biosynthesis of the polyketide albicidin in *Xanthomonas albilineans* (Vivien *et al.*, 2005).

In previous studies in our laboratory, de Bruijn & Raaijmakers (de Bruijn & Raaijmakers, 2009b) identified the serine protease ClpP as a regulator of massetolide biosynthesis in strain *P. fluorescens* SS101. The ATP-dependent serine protease ClpP is highly conserved in eubacteria (Maurizi *et al.*, 1990) and has diverse functions, including intracellular protein degradation. At the transcriptional level, ClpP-mediated regulation of massetolide biosynthesis appeared to function independently from the two-component regulation by GacS/GacA (de Bruijn & Raaijmakers, 2009b). Moreover, site-directed mutagenesis of the chaperon endoing gene *clpX* did not affect massetolide biosynthesis (de Bruijn & Raaijmakers, 2009b), suggesting that ClpX does not act as a chaperon of ClpP in the regulation of massetolide biosynthesis. Based on these findings a model was proposed in which ClpP regulates, alone or together with a yet unknown chaperone, massetolide biosynthesis via degradation of putative transcriptional repressors of the LuxR-type transcriptional regulator *massAR* and/or via modulation of the citric acid cycle and amino acid metabolism (de Bruijn & Raaijmakers, 2009b).

So far, most of the identified regulatory genes of LP biosynthesis were found based on screening for LP deficiency. There is also a study showing that certain genes negatively regulate LP production. In *Pseudomonas* sp. strain DF41, *relA* and *spoT* are two genes known to be involved in lipopeptide production. RelA is a synthase that generates (p) ppGpp when there is limited amino acids availability. SpoT can act either as a hydrolase or as a synthase depending on the conditions (Potrykus & Cashel, 2008). HPLC analysis of culture extracts of both Δ*relA* and Δ*relAspoT* mutants revealed that compared to wild type, production of the LP sclerosin was enhanced by 1.5-2.0 fold. This corresponded with a 5-fold increased expression of the LP sclerosin biosynthetic genes in the mutants as compared to the wild type (Manuel *et al.*, 2011). In other strains, however, mutations in *spoT* led to reduced LP production as was the case for arthrofactin-producing *Pseudomonas* sp. strain MIS38 (Washio et al., 2010).

Natural functions of lipopeptides

LPs produced by *Pseudomonas* species exhibit lytic and growth-inhibitory activities against a broad range of microorganisms, including viruses, mycoplasmas, bacteria, fungi and oomycetes. In plant-associated *Pseudomonas* strains, LPs also play a role in colonization of seeds (Nielsen *et al.*, 2005) and roots (Tran *et al.*, 2007), in defense against competing microorganisms and predatory protozoa (Mazzola *et al.*, 2009), and in swarming motility and biofilm formation (Raaijmakers et al., 2010). Below I briefly summarize the natural functions of LPs.

Antiviral and antibacterial activities

The LP viscosin was reported for its antiviral activity against enveloped viruses (reviewed in Nybroe & Sørensen, 2004). Antibacterial activities were observed for several LPs. Massetolides, viscosin, syringopeptin and syringomycins showed activity against Gram-positive *Mycobacterium tuberculosis*, *Mycobacterium avium-intercellulare* and *Mycobacterium smegmatis* (Gerard *et al.*, 1997, El Sayed *et al.*, 2000, Buber *et al.*, 2002). In a recent study, brabantamides A–C also displayed moderate to high *in vitro* activities against Gram-positive bacterial pathogens (Schmidt et al., 2014). On the other hand, very few of the tested LPs have activity against Gram-negative bacteria. This has been attributed to the outer membrane of Gram-negative bacteria which might hinder the access of LPs to the plasma membrane (Nybroe & Sørensen, 2004). However, the antimicrobial activity of xantholysins is not confined to Gram-positive bacteria, but also extends to some Gram-negative strains, including *Xanthomonas* (Li et al., 2013). The same inhibitory effect was also shown for WLIP against *Xanthomonas* (Rokni-Zadeh et al., 2012), although the underlying mechanisms remain elusive.

Antifungal and anti-oomycetal activities

Antifungal activities have been described for many different LPs (Raaijmakers et al., 2006, Ongena & Jacques, 2008). For a few LPs, more detailed investigations were carried out to elucidate their effects on fungal cell morphology and physiology. For instance, when supplementing LP tensin in the agar medium, mycelium of *Rhizoctonia solani* showed retarded growth accompanied by increased branching and rosette formation as well as hyphal swellings (Nielsen et al., 2000). Similar phenotypic effects as well as the development of aerial hyphae were observed for viscosinamide (Nielsen et al., 1999, Thrane *et al.*, 1999). These effects might be caused by increased Ca^{2+} and H^{+} influx in target cells which may or may not be associated with the ability of LPs to form pores in the cell membrane (Thrane et al., 1999).

Zoospores of oomycetes such as *Pythium* and *Phytophthora* species can be lysed by LPs. So far, viscosin, viscosinamide, massetolide A, putisolvins and orfamide A have been well-characterized for their impact on zoospores. At low concentrations (~5 µg mL^{-1}), massetolide A and viscosinamide did not lyse zoospores but induced encystment of zoospores of *Phytophthora infestans* and *Pythium* sp. P11, respectively (Thrane *et al.*, 2000, van de Mortel *et al.*, 2009). On the other hand, at higher concentrations (~25 µg mL^{-1}), massetolide A, putisolvins and orfamide immobilize zoospores from different oomycetes and cause lysis of entire zoospore populations within 1 min (de Souza *et al.*, 2003a, de Souza *et al.*, 2003b, Gross et al., 2007, Tran *et al.*, 2008, Kruijt *et al.*, 2009, van de Mortel et al., 2009).

Anti-predation

The plant rhizosphere is home to high numbers of microorganisms, which leads to increases in the populations and feeding activities of their predators (Taylor, 1978). Predation plays a significant role in shaping the structure of bacterial communities (Ronn *et al.*, 2002, Bonkowski & Brandt, 2002). Bacteria possess various defense strategies to evade predation by protozoa via both intracellular and extracellular adaptations (Matz & Kjelleberg, 2005). For *Pseudomonas* species, hydrogen cyanide (HCN), 2,4-diacetylphloroglucinol (2,4-DAPG) and pyrrolnitrin (PRN) were shown to contribute to defense against protozoa (Jousset *et al.*, 2010, Gallagher & Manoil, 2001). Also extracellular proteases inhibit protozoan predation in *Pseudomonas* (Jousset *et al.*, 2006) as well as in *Vibrio cholerae* (Vaitkevicius *et al.*, 2006, Niu *et al.*, 2010). Mazzola et al (2009) assessed the function of LPs in defense against protozoan predation and showed that the LPs massetolide A and viscosin limit protozoan grazing both *in vitro* and *in situ*. Interestingly, protozoa-*Pseudomonas* interactions led to enhanced transcription of LP biosynthesis genes (Mazzola et al., 2009). These results suggested that bacteria can modulate production of secondary metabolites in response to the presence of protozoan predators.

Motility

Motility of bacteria has been extensively studied, including swimming, swarming and twitching (Henrichsen, 1972). During swarming, vegetative cells of bacteria can differentiate into hyperflagellated swarmer cells which are generally longer (Harshey, 2003). To address the role of LPs in motility of *Pseudomonas*, LP-deficient mutants were generated and their surface motility tested *in vitro* on semi-solid agar plates. In most cases, surface motility was lost or reduced in the LP-deficient mutants (Table 2). However, in one case, mutations in genes coding for biosynthesis of the LP sessillin in *Pseudomonas* CMR12a caused increased motility (D'Aes et al., 2014). Supplementing purified LP to the medium generally restores surface motility in LP-deficient mutants (Andersen *et al.*, 2003, de Bruijn et al., 2007). As yet, it is not known if LPs also contribute to dispersal in natural habitats. Tran et al (2007) showed that wild-type *P. fluorescens* SS101, when applied to tomato seeds, was more effective in colonization of the root system of tomato seedlings than its LP-deficient mutant. Similarly, the viscosin-deficient mutant of plant pathogenic *P. fluorescens* strain 5064 was unable to colonize the surface of intact broccoli florets to the same extent as its wild type (Hildebrand *et al.*, 1998). Amphisin produced by *Pseudomonas* species DSS73, was also shown to be essential for colonization of sugar beet on seeds (Nielsen et al., 2005).

Table 2. Involvement of lipopeptides in motility and biofilm formation of *Pseudomonas*

Strains	Lipopeptides	Motility in LPs mutant	Biofilm in LPs mutant	References
Pseudomonas fluorescens SS101	massetolide A	Lost	Reduced	de Bruijn et al., 2008
Pseudomonas fluorescens SBW25	viscosin	Lost	Reduced	de Bruijn et al., 2007
Pseudomonas protegens Pf-5	orfamide	Reduced	No change	Gross et al., 2007
Pseudomonas CMR12a	orfamide	Lost	Reduced	D'Aes et al., 2014
Pseudomonas CMR12a	sesillin	Increased	Reduced	D'Aes et al., 2014
Pseudomonas putida	putisolvin	Reduced	Increased	Dubern et al., 2008
Pseudomonas species MIS38	arthrofactin	Lost	Increased	Roongsawang et al., 2003
Pseudomonas species DSS73	amphisin	Lost		Andersen et al., 2003
Pseudomonas syringae pv. tomato	syringafactin	Lost		Berti et al., 2007
Pseudomonas putida RW10S2	WLIP	Lost	Reduced	Rokni-Zadeh et al., 2012
Pseudomonas putida BW11M1	xantholysin	Lost	Reduced	Li et al., 2013
Pseudomonas cichorii SF1-54	cichofactin	Lost	Increased	Pauwelyn et al., 2013

Biofilm formation

For *Pseudomonas*, LPs play an important role in surface attachment and biofilm formation, albeit with different outcomes depending on the type of LP (Raaijmakers et al., 2010). In most cases, biofilm formation was reduced in the LP-deficient mutants (Table 2). However, in some LP deficient mutants, biofilm formation increased or did not change compared to that of the wild type. For example, anthrofactin-producing *Pseudomonas* MIS38 forms a biofilm, whereas arthrofactin-deficient mutants form unstable, but more biofilms (Roongsawang et al., 2003). Similar results were found for putisolvin- and cichofactin-producing *Pseudomonas* (Kuiper *et al.*, 2004, Kruijt et al., 2009, Pauwelyn et al., 2013). On the other hand, for *P. fluorescens* SS101 and SBW25, *Pseudomonas* CMR12a, *P. putida* RW10S2, *P. putida* BW11M1, mutants deficient in the LPs massetolide, viscosin, sessilin, WLIP and xantholysin formed significantly less biofilm (Table 2) (de Bruijn et al., 2007, de Bruijn et al., 2008, D'Aes et al., 2014, Rokni-Zadeh et al., 2012, Li et al., 2013). The diversity in structures and hydrophobicities of the LPs might result in different roles in biofilm formation (de Bruijn et al., 2008).

Outline of this thesis

The **overall aim of my PhD research** was to elucidate the regulatory pathways of LP biosynthesis and to unravel the natural functions of LPs produced by *Pseudomonas* species. This thesis focused specifically on LPs produced by three different strains in the *P. fluorescens* group. The first strain is *P. fluorescens* SS101, which was originally isolated from the rhizosphere of wheat (de Souza et al., 2003a) and has activity against various oomycete and fungal pathogens (de Souza et al., 2003a, Tran et al., 2007, van de Mortel et al., 2009). The LP produced by strain SS101 is massetolide A (de Bruijn et al., 2008). The second strain studied in this thesis is *P. protegens* Pf-5 which was isolated from cotton rhizosphere and produces a series of secondary metabolites and the LP orfamide (Gross et al., 2007). The third one is *P. fluorescens* SBW25. It was first isolated from the leaf surface of sugar beet (Deleij *et al.*, 1995). Since then it has been extensively studied for its plant growth-promoting properties and biocontrol potential to suppress seedling damping-off diseases (Naseby *et al.*, 2001). The LP produced by SBW25 is viscosin (de Bruijn et al., 2007).

In **Chapter 2**, we conducted a genome-wide search for small RNAs (sRNAs) in *P. fluorescens* SS101 and performed transcriptomic analyses to identify genes associated with the Rsm (repressor of secondary metabolites) regulon. We addressed the significance of the Rsm regulon, and in particular that of the two small RNAs RsmY (PflSS101_4962) and RsmZ (PflSS101_1168) and the two repressor proteins RsmA (PflSS101_4138) and RsmE (PflSS101_3491), in massetolide biosynthesis in SS101 and predicted the potential target genes of the Rsm repressor proteins. Via transcriptome, mutational and phenotypic analyses, we showed that the Rsm system regulates massetolide biosynthesis as well as the expression of several other genes and traits in *P. fluorescens* SS101.

In **Chapter 3** we described the results of a genome-wide search for new regulatory genes of massetolide biosynthesis in *P. fluorescens* SS101. Screening of two independent random plasposon mutant libraries (~8,000 mutants total) for a reduced or loss of massetolide production resulted in thirteen putative regulatory mutants. Further analyses of the mutants led to the identification of putative regulatory genes of massetolide biosynthesis, namely *prtR*, *phgdh*, *dnaK* and *clpA*. Genetic, phenotypic, chemical and transcriptional analyses were performed to elucidate the functions of *prtR*, *phgdh*, and *dnaK* in massetolide biosynthesis and in other phenotypic traits, including swarming motility, siderophore production and extracellular protease activity.

In **Chapter 4**, the role of ClpA in the regulation of massetolide biosynthesis was investigated in more detail. ClpA is one of the ATPases associated with ClpP, a serine protease involved in intracellular proteolysis. Transcriptomic and proteomic analyses were conducted for both *clpA* and *clpP* mutants with the ultimate goal to identify genes and proteins that are part of ClpAP-mediated regulation of massetolide biosynthesis. The results show that ClpAP complex regulates massetolide biosynthesis via the

pathway-specific, LuxR-type regulator MassAR, the heat shock proteins DnaK and DnaJ, and proteins of the tricarboxylic acid (TCA) cycle.

In **Chapter 5**, experiments were conducted to unravel the role of LPs of different *P. fluorescens* strains in defense against protozoan predation. To that end, whole-genome transcriptome analysis, MALDI-TOF-based imaging mass spectrometry (IMS) and live colony NanoDESI mass spectrometry were conducted to monitor *in situ* changes in gene expression and production of metabolites at the interface of protozoa-*Pseudomonas* interaction. This investigation specifically focussed on the role of LPs in defence against protozoan predation but also provided additional insights into the chemical interplay between the predator and the prey.

In **Chapter 6**, the role of the LP orfamide in swarming motility of *P. protegens* Pf-5 was investigated in detail. We observed that two orfamide-deficient mutants of Pf-5, with deletions in either the orfamide biosynthesis gene *ofaA* or in the transcriptional regulatory gene *gacA*, 'hitch-hike' with their parental strain under swarming conditions. Both the *ofaA* and the *gacA* mutant behave as social cheaters with respect to swarming motility. However, the two mutants exhibit a distinctly different spatial distribution, with the *gacA* mutant predominating on the edge of the co-swarming colonies. Subsequent experimental evolution assays with wild type Pf-5 showed that social cheaters accumulate on the edge of Pf-5 colonies during successive swarming. The vast majority of these social cheaters had mutations inactivating the GacS/GacA two-component regulatory system. Genetic, phenotypic, microscopic and whole-genome transcriptomic analyses were conducted to assess the fitness benefits of these social cheaters that arise spontaneously during successive swarming.

In **Chapter 7**, the most important findings of my thesis are summarized and suggestions for future research are discussed.

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Chapter 2

The Rsm regulon of plant growth-promoting *Pseudomonas fluorescens* SS101: role of small RNAs in regulation of lipopeptide biosynthesis

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Summary

The rhizobacterium *Pseudomonas fluorescens* SS101 inhibits growth of oomycete and fungal pathogens, and induces resistance in plants against pathogens and insects. To unravel regulatory pathways of secondary metabolite production in SS101, we conducted a genome-wide search for sRNAs and performed transcriptomic analyses to identify genes associated with the Rsm (repressor of secondary metabolites) regulon. *In silico* analysis led to the identification of sixteen putative sRNAs in the SS101 genome. In frame deletion of the sRNAs *rsmY* and *rsmZ* showed that the Rsm system regulates the biosynthesis of the lipopeptide massetolide A and involves the two repressor proteins RsmA and RsmE, with the LuxR-type transcriptional regulator MassAR as their most likely target. Transcriptome analyses of the *rsmYZ* mutant further revealed that genes associated with iron acquisition, motility and chemotaxis were significantly upregulated, whereas genes of the type VI secretion system were downregulated. Comparative transcriptomic analyses showed that most, but not all, of the genes controlled by RsmY/RsmZ are also controlled by the GacS/GacA two-component system. We conclude that the Rsm regulon of *P. fluorescens* SS101 plays a critical role in the regulation of lipopeptide biosynthesis and controls the expression of other genes involved in motility, competition and survival in the plant rhizosphere.

Introduction

Computational searches of intergenic regions, promoters and rho-independent transcription terminators (Livny *et al.*, 2005, Livny *et al.*, 2006, Sridhar & Gunasekaran, 2013, Wright *et al.*, 2013) combined with experimental approaches (Sharma & Vogel, 2009) have revealed the presence of several small RNAs (sRNAs) in bacterial genomes. In general, two types of regulatory sRNAs have been described (Majdalani *et al.*, 2005, Gottesman *et al.*, 2006, Pichon & Felden, 2007, Gottesman & Storz, 2011). The first targets specific mRNAs by base-pairing. An example is RyhB in *E. coli* which interacts with the mRNA encoding SodB, an iron-containing superoxide dismutase (Salvail *et al.*, 2010). The second type interacts with RNA-binding proteins of the RsmA/CsrA family. RsmA (regulator of secondary metabolism) and CsrA (carbon storage regulator) act as translational repressors and their sequestration by activated sRNAs can relieve repression of the target mRNAs.

In *Pseudomonas*, relatively few sRNAs have been studied in detail for their functions. In *Pseudomonas protegens* strain CHA0, the sRNAs RsmX, RsmY and RsmZ are under the control of the GacS/GacA two-component system and regulate the production of a range of secondary metabolites (Heeb *et al.*, 2002b, Valverde *et al.*, 2003, Kay *et al.*, 2005, Lapouge *et al.*, 2007, Lapouge *et al.*, 2008). In *P. protegens* CHA0, Gac/Rsm-mediated regulation of secondary metabolites involves sequestration of the repressor proteins RsmA and RsmE that act post-transcriptionally by binding to the target mRNA (Blumer *et al.*, 1999, Reimmann *et al.*, 2005, Lapouge et al., 2008). In *Pseudomonas aeruginosa*, the two sRNAs RsmY and RsmZ regulate quorum sensing and the biosynthesis of several exoproducts (Brencic *et al.*, 2009, Frangipani *et al.*, 2014). Other sRNAs described for *P. aeruginosa* are PhrS, PrrF1 and PrrF2: PhrS is involved in the regulation of quinolone biosynthesis (Sonnleitner *et al.*, 2011), and PrrF1 and PrrF2 contribute to iron acquisition (Wilderman *et al.*, 2004, Sonnleitner & Haas, 2011).

Most of the known sRNAs in *Pseudomonas* and other Gram-negative bacterial genera are under the control of the Gac/Rsm signal transduction pathway. Based on the proposed model, the phosphorylated regulator GacA binds to a conserved element upstream of the sRNA promoter, referred to as the GacA box, to activate their expression (Lapouge et al., 2008). In many cases, mutations or deletions of the sRNAs result in phenotypes similar to that of GacS/GacA mutants. For example, Δ*rsmYZ* and Δ*gacA* mutants of *Pseudomonas aeruginosa* are both deficient in the synthesis of the quorum sensing signal N-butanoyl-homoserine lactone, hydrogen cyanide, pyocyanin, elastase, and chitinase as well as in biofilm formation (Kay *et al.*, 2006, Brencic et al., 2009). In *Pseudomonas entomophila*, Δ*rsmYZ* and Δ*gacA* mutants were both deficient in the production of entolysin (Vallet-Gely *et al.*, 2010). Similarities in phenotypes of *rsm* and *gac* mutants have also been described for *Pectobacterium carotovorum* (Liu *et al.*, 1998), *Escherichia coli* (Weilbacher *et al.*, 2003), *Salmonella enterica* (Fortune *et al.*, 2006), and *Legionella pneumophila* (Sahr *et al.*, 2009).

In this study, we conducted a genome-wide search for sRNAs in *Pseudomonas fluorescens* strain SS101 and performed transcriptomic analyses to identify genes associated with the Rsm regulon and with the Gac regulon. We addressed the function of the Rsm regulon, involving the two sRNAs RsmY (PflSS101_4962) and RsmZ (PflSS101_1168), and the two repressor proteins RsmA (PflSS101_4138), and RsmE (PflSS101_3491), in lipopeptide biosynthesis and predicted the potential target genes of the Rsm repressor proteins. Strain SS101 was originally isolated from the rhizosphere of wheat (de Souza *et al.*, 2003), has activity against various oomycete and fungal pathogens (de Souza et al., 2003, Tran *et al.*, 2007, van de Mortel *et al.*, 2009), and induces systemic resistance in tomato and Arabidopsis against several pathogens and insect pests (Tran et al., 2007, van de Mortel *et al.*, 2012). Comparative genome analyses of multiple *Pseudomonas* species and strains (Loper *et al.*, 2012) revealed that strain SS101 harbours 350 unique genes, which include prophage and genomic islands. Unlike many other *P. fluorescens* and *P. protegens* biocontrol strains, SS101 does not produce the typical secondary metabolites such as 2,4-diacetylphloroglucinol (DAPG), phenazines, pyrrolnitrin, pyoluteorin and hydrogen cyanide (HCN) (Loper et al., 2012). The main secondary metabolite produced by SS101 is the cyclic lipopeptide massetolide A, whose biosynthesis is governed by the nonribosomal peptide synthetase (NRPS) genes *massABC* and regulated by the GacS/GacA system (de Bruijn & Raaijmakers, 2009b). Massetolide A contributes to biofilm formation, swarming motility, antimicrobial activity and defense against protozoan predators (Mazzola *et al.*, 2009, Raaijmakers *et al.*, 2010). Here, genome-wide transcriptional analysis of mutants with deletions in *rsmY* and *rsmZ* revealed that the NRPS genes *massA*, *massB*, *massC* as well as the LuxR-type transcriptional regulator *massAR* were significantly down-regulated. Via mutational and phenotypic analyses, we show that the Rsm system regulates massetolide biosynthesis as well as several other genes and traits in the rhizobacterium *P. fluorescens* SS101.

Results and Discussion

Small RNAs in *Pseudomonas fluorescens* SS101

A total of 68 tRNAs and 19 rRNAs were found in the SS101 genome (Table S1). Genome-wide analyses revealed sixteen predicted sRNAs including homologues of the two signal recognition particle RNAs SrpB_1 (PflSS101_3911) and SrpB_2 (PflSS101_3926) (Table 1). Srp is a ribonucleoprotein complex that participates in multiple protein targeting pathways in bacteria (Koch *et al.*, 1999) and is primarily involved in the incorporation of proteins in the inner membrane (Rosenblad *et al.*, 2009). Furthermore, we also found a 6S SsrS RNA (PflSS101_5226) in the SS101 genome. In *E. coli*, 6S RNA is encoded by the *ssrS* gene which regulates transcription during late exponential and stationary growth (Wassarman, 2007). Bacterial RNase P (Ribonuclease P) (PflSS101_0956) was found in the SS101 genome and represents a ribonucleoprotein complex comprised of a single RNA (~400 nt) and a single small protein subunit (~14 kDa) with the RNA as the catalytic subunit of the enzyme involved in the maturation of tRNA transcripts (Ellis & Brown,

2009). We also found homologues of PhrS (PflSS101_4081), PrrF1 (PflSS101_4589) and PrrF2 (PflSS101_3274), which are known to repress or activate the translation of target mRNAs by a base-pairing mechanism. In *P. aeruginosa*, the two *prrF* sRNA genes are found in tandem. Homologous genes in other *Pseudomonas* species are located considerably distant from each other on the chromosome (Wilderman et al., 2004). Also in SS101, PrrF1 (PflSS101_4589) and PrrF2 (PflSS101_3274) are found at different locations in the genome. We also found RgsA (PflSS101_1357) in the SS101 genome, which is a sRNA probably regulated indirectly by GacA and directly by the stress sigma factor RpoS (Gonzalez *et al.*, 2008).

Two other sRNAs found in the SS101 genome were RsmY (PflSS101_4962) and RsmZ (PflSS101_1168) (Table 1). In *P. protegens* and *P. aeruginosa*, RsmY and RsmZ regulate secondary metabolite production by sequestering RNA-binding proteins (e.g. CsrA, RsmA) that act as translational repressors (Kay et al., 2005, Gottesman & Storz, 2011). In *P. aeruginosa*, the expression of all Gac-regulated genes was shown to be RsmY/Z-dependent (Brencic et al., 2009). For the other sRNAs detected in the SS101 genome (Table 1), the functions are poorly understood or not known from other *Pseudomonas* species. Here, we will specifically focus on the sRNAs in strain SS101 that are regulated by the GacS/GacA two-component system.

Table 1. Small non-coding RNAs in *Pseudomonas fluorescens* SS101

Gene Locus	Small RNAs descriptions	Fold change in Δ*gacS*[a]	P value	Fold change in Δ*gacA*[a]	P value
PflSS101_0956	Bacterial RNase P class A	1.46	0.0428	1.37	0.17
PflSS101_1168	RsmZ RNA	-27.43	6.46E-06	-21.94	1.11E-05
PflSS101_1276	putative t44 RNA	-1.41	0.00672	-1.29	0.0135
PflSS101_1357	RgsA RNA	-1.56	0.0206	-1.53	0.016
PflSS101_2033	putative sRNA P15	-1.06	0.865	-1.01	0.965
PflSS101_3274	PrrF2 RNA	1.69	0.00185	1.52	0.00528
PflSS101_3911	srpB_1: Bacterial signal recognition particle RNA	1.14	0.772	-1.02	0.965
PflSS101_3926	srpB_2: Bacterial signal recognition particle RNA	1.32	0.257	1.29	0.319
PflSS101_3951	sRNA P11	-1.16	0.615	-1.1	0.702
PflSS101_4081	PhrS RNA	1.23	0.0335	1.29	0.0359
PflSS101_4589	PrrF1 RNA	6.05	0.000153	5.88	0.000479
PflSS101_4738	sRNA P24	1.29	0.0629	1.19	0.392
PflSS101_4885	sRNA P26	-1.17	0.399	-1.35	0.126
PflSS101_4962	RsmY RNA	-3.44	3.78E-06	-3.22	5.56E-05
PflSS101_5194	sRNA P1	1.76	0.0314	1.26	0.289
PflSS101_5226	6S SsrS RNA	1.92	0.0112	2.25	0.00617

All predicted small non-coding RNAs in *P. fluorescens* SS101 are indicated.

a. Positive values correspond to higher expression, negative values to lower expression (compared to the wild type). The sRNAs for which the expression is statistically significant (Fold change>=2; P< 0.001) in both the Δ*gacS* and Δ*gacA* mutant vs. wild type SS101 are shaded in grey.

Small RNAs in *Pseudomonas fluorescens* SS101 regulated by the GacS/A system

Transcriptomic analyses of both *gacS* and *gacA* mutants of *P. fluorescens* SS101 (Table S2, S3) revealed that the expression of three sRNAs (*rsmY, rsmZ* and *prrF1*) was significantly (fold change (FC) >2-fold; P<0.001) altered (Table 1). Expression of *rsmY* and *rsmZ* was significantly down-regulated in both *gacS* and *gacA* mutants, whereas expression of *prrF1* was approximately 6-fold upregulated in both *gac* mutants. The predicted sizes of the *rsmY, rsmZ* and *prrF1* transcripts were 118, 133, and 112 bp, respectively. Subsequent prediction of their secondary structures revealed 8 GGA motifs in both RsmY and RsmZ, with three in predicted loop regions, respectively (Figure 1A). In contrast, only one GGA motif was found in PrrF1, which is localized to a predicted stem (Figure 1A). Repeated GGA motifs in loop regions of the secondary structure, as predicted for RsmY and RsmZ, are an essential characteristic of sRNAs for sequestration of RsmA and homologous repressor proteins (Lapouge et al., 2008). Previous work also showed that the regions upstream of these sRNAs contain a conserved 18-bp sequence which corresponds to the GacA-binding site for activation of these sRNAs (Heeb *et al.*, 2002a, Kay et al., 2005). For SS101, we indeed found this typical GacA-binding box upstream of *rsmY* and *rsmZ* (Figure 1B, 1C), but not for *prrF1*. Therefore, our subsequent functional analyses focused on *rsmY* and *rsmZ*.

Role of RsmY and RsmZ in lipopeptide biosynthesis in *P. fluorescens* SS101

The location of *rsmY* and *rsmZ* in the genomes appears to be conserved, at least to some extent, for the different *Pseudomonas* species and strains (Figure 1B, 1C). In frame deletion mutants were generated to investigate the role of *rsmY* and *rsmZ* in the regulation of massetolide A biosynthesis. The drop collapse assay, a reliable proxy for detection of massetolide A and other lipopeptide surfactants (de Bruijn *et al.*, 2008, de Bruijn & Raaijmakers, 2009b), showed that mutations in either *rsmY* or *rsmZ* alone did not affect massetolide A production (Figure 2A). However, mutations in both *rsmY* and *rsmZ* resulted in loss of massetolide A production which was confirmed by RP-HPLC (Figure 2B). Also swarming motility of SS101, a phenotype that depends on massetolide production (de Bruijn et al., 2008), was abolished in the *rsmYZ* double mutant (Figure 2C). Mutations in *rsmY* or *rsmZ* alone did not affect growth of strain SS101 (Figure 2D). However, mutations in both *rsmY* and *rsmZ* slightly enhanced growth in the early exponential phase but had an adverse effect on growth during the late exponential and stationary phase; similar changes in growth dynamics were observed for the *gacS* and *gacA* mutants of strain SS101 (Figure 2D). These changes in growth dynamics are most likely not related to a lack of massetolide production, because growth of the site-directed *massA* biosynthesis mutant of SS101 was similar to that of the wild type (de Bruijn & Raaijmakers, 2009b). In summary, these results indicated that both RsmY and RsmZ are an integral component of the GacS/GacA signal transduction cascade and regulate massetolide biosynthesis in *P. fluorescens* SS101.

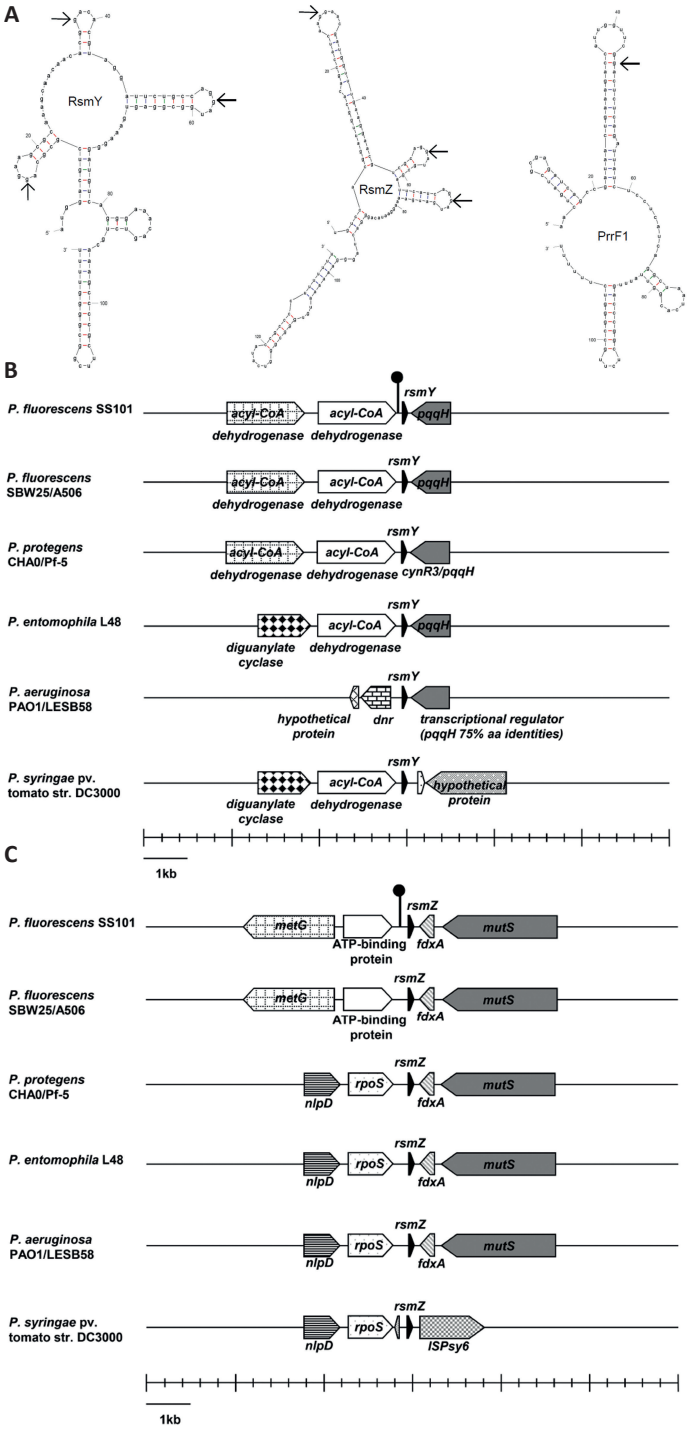

Figure 1. Secondary structures of small RNAs, RsmY, RsmZ, PrrF1 in *Pseudomonas fluorescens* SS101 and the genetic organization of *rsmY* and *rsmZ* in strain SS101 and other *Pseudomonas* species and strains.

(A). Predicted secondary structures of RsmY, RsmZ and PrrF1 of *P. fluorescens* SS101 by MFOLD (http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form). The typical GGA motifs located in the loop regions are indicated with arrows.

(B). Genetic organization of *rsmY* regions in different *Pseudomonas* species and strains. Block arrows indicate directionality of the open reading frame, and orthologous genes are represented by color and pattern. The loop symbol in front of *rsmY* indicates the position of the upstream activating sequence (UAS for *rsmY*: TGTAAGCATTCTCTTACA). Abbreviations: *pqqH /cynR3/dnr*: transcriptional regulator.

(C). Genetic organization of *rsmZ* regions in different *Pseudomonas* species and strains. Block arrows indicate directionality of the open reading frame, and orthologous genes are represented by color and pattern. The loop symbol in front of *rsmZ* indicates the position of the UAS (UAS for *rsmZ*: TGTAAGCATTCGCTTACT). Abbreviations: *metG*: methionyl-tRNA synthetase; *fdxA*: ferredoxin; *mutS*: DNA mismatch repair protein; *nlpD*: lipoprotein; *rpoS*: RNA polymerase sigma factor; ISPsy6: transposase.

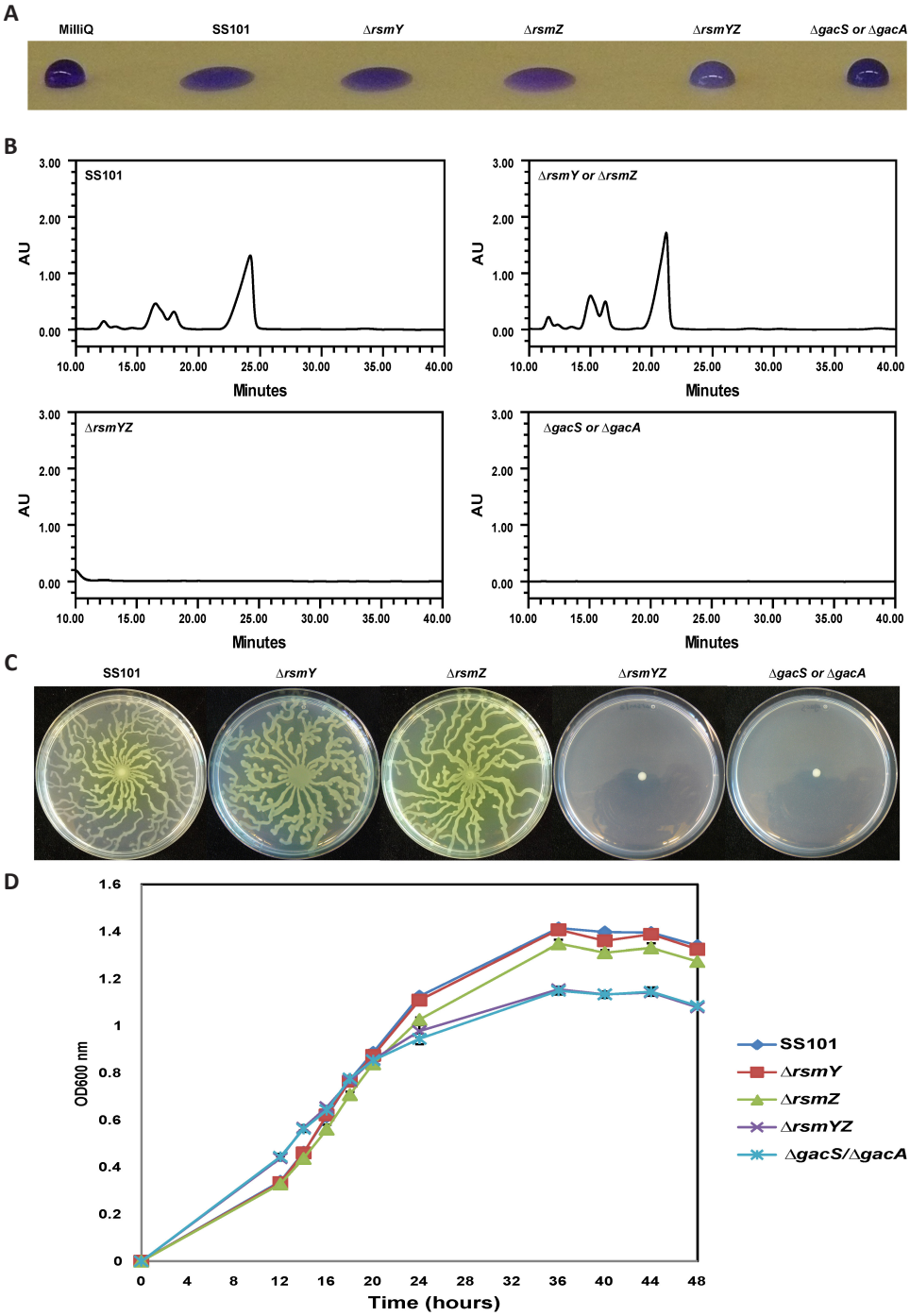

Figure 2. Phenotypic and chemical analyses of *Pseudomonas fluorescens* strain SS101, and single or double mutants disrupted in *rsmY*, *rsmZ*, *gacS* or *gacA*.
(A) Drop collapse assay with cell cultures of wild-type strain SS101, Δ*rsmY*, Δ*rsmZ*, Δ*rsmYZ*, Δ*gacS* and Δ*gacA*

mutants. Bacterial cultures grown for 2 days at 25°C on KB agar plates were suspended in sterile water to a final density of $1x10^{10}$ cells/ml, and 10-µl droplets were spotted on parafilm and crystal violet was added to the droplets to facilitate visual assessment. A flat droplet is a highly reliable proxy for the production of the surface-active lipopeptide massetolide A.
(B) RP-HPLC chromatograms of cell-free culture extracts of wild-type strain SS101, Δ*rsmY*, Δ*rsmZ*, Δ*rsmYZ*, Δ*gacS* and Δ*gacA* mutants as described in panel A. The wild-type strain SS101 produces massetolide A (retention time of approximately 23-25 min) and various other derivatives of massetolide A (minor peaks with retention times ranging from 12 to 18 min) which differ from massetolide A in the amino acid composition of the peptide moiety. AU stands for Absorbance Unit.
(C) Swarming motility of wild-type strain SS101, Δ*rsmY*, Δ*rsmZ*, Δ*rsmYZ*, Δ*gacS* and Δ*gacA* mutants on soft (0.6% [wt/vol]) agar plates. Five microliters ($1x10^{10}$ cells/ml) of washed overnight cultures of wild-type SS101 or mutants were spot-inoculated in the center of a soft agar plate and incubated for 48 to 72 h at 25°C.
(D) Growth of wild-type strain SS101, Δ*rsmY*, Δ*rsmZ*, Δ*rsmYZ*, Δ*gacS* and Δ*gacA* mutants in liquid broth at 25°C. At different time points, the optical density of the cell cultures was measured spectrophotometrically (600 nm). Mean values of four biological replicates are given; the error bars represent the standard error of the mean.

Deletion of repressor proteins restores massetolide production

Previous studies with *P. protegens* CHA0 have shown that Gac/Rsm-mediated regulation of secondary metabolites involves sequestration of the repressor proteins RsmA and RsmE that act post-transcriptionally by binding to the target mRNA (Blumer et al., 1999, Reimmann et al., 2005, Lapouge et al., 2008). Hence, the next step was to determine if these repressor proteins are present in SS101 and if they play a role in Gac/Rsm-mediated regulation of massetolide biosynthesis. *In silico* analysis of the SS101 genome led to the identification of *rsmA* (PflSS101_4138), *rsmE* (PflSS101_3491) and *csrA* (PflSS101_3653). Phylogenetic analyses showed that they clustered closely with their homologues in other *P. fluorescens* strains and *Pseudomonas* species at both DNA and protein levels (Figure S1). To decipher their role in regulation of massetolide biosynthesis, deletion mutants were made for each of these three repressors in the *gacS* mutant background of strain SS101. The *gacS* mutant does not produce massetolide, but according to the regulatory model, a mutation of the repressor proteins would alleviate translational repression and restore production. The results of the drop collapse assay and RP-HPLC analyses showed that a deletion of either *rsmA* or *csrA* in the *gacS* mutant did not restore massetolide production (Figure 3A; 3B). Based on the drop collapse assay, a mutation in the *rsmE* gene partially affected the surface tension (Figure 3A) but massetolide production was not detectable by RP-HPLC analysis (Figure 3B). A double mutation in *rsmE* and *rsmA* fully restored massetolide production (Figure 3A, 3B). A single deletion of either one of the repressor genes did not affect growth as compared to that of the *gacS* mutant, whereas stacked deletions of *rsmA* and *rsmE* in the *gacS* mutant changed the growth dynamics back to that of the wild type (Figure 3C). We conclude that Gac/Rsm-mediated regulation of massetolide biosynthesis via *rsmY* and *rsmZ* implicates the two small RNA binding proteins RsmA and RsmE, whereas CsrA is not involved.

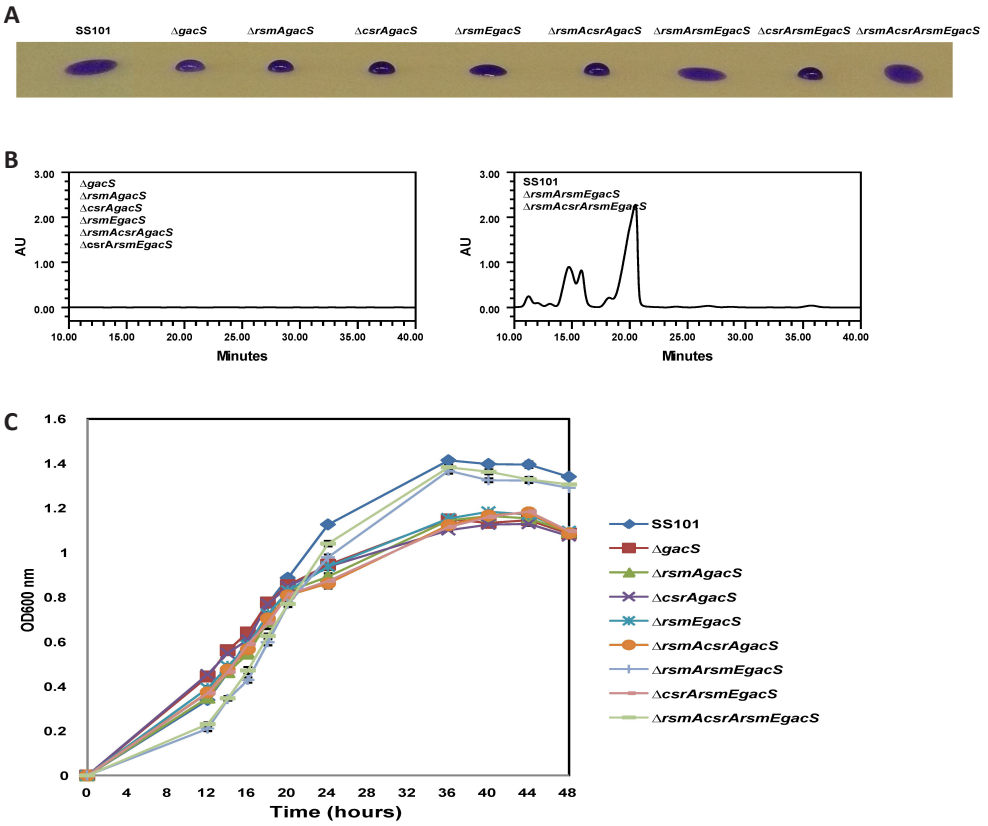

Figure 3. Phenotypic and chemical analyses of *Pseudomonas fluorescens* strain SS101, Δ*gacS* mutant, and single, double or triple mutants disrupted in *rsmA*, *rsmE* and *csrA* in the Δ*gacS* background.
(A) Drop collapse assay with cell suspensions of wild-type SS101, Δ*gacS*, Δ*rsmAgacS*, Δ*csrAgacS*, Δ*rsmEgacS*, Δ*rsmAcsrAgacS*, Δ*rsmArsmEgacS*, Δ*csrArsmEgacS* and Δ*rsmAcsrArsmEgacS* mutants. Bacterial cultures grown for 2 days at 25°C on KB agar plates were suspended in sterile water to a final density of $1x10^{10}$ cells/ml and 10-µl droplets were spotted on parafilm and crystal violet was added to the droplets to facilitate visual assessment. A flat droplet is a highly reliable proxy for the production of the surface-active lipopeptide massetolide A.
(B) RP-HPLC chromatograms of cell-free culture extracts of wild-type SS101, Δ*rsmAgacS*, Δ*csrAgacS*, Δ*rsmEgacS*, Δ*rsmAcsrAgacS*, Δ*rsmArsmEgacS*, Δ*csrArsmEgacS*, Δ*rsmAcsrArsmEgacS* mutants as described in panel A. The wild-type strain SS101 produces massetolide A (retention time of approximately 18-21 min) and various other derivatives of massetolide A (minor peaks with retention times ranging from 12 to 18 min) which differ from massetolide A in the amino acid composition of the peptide moiety. AU stands for Absorbance Unit. Representative chromatograms of Δ*rsmAgacS* and Δ*rsmArsmEgacS* mutants are shown.
(C) Growth of wild-type SS101, Δ*rsmAgacS*, Δ*csrAgacS*, Δ*rsmEgacS*, Δ*rsmAcsrAgacS*, Δ*rsmArsmEgacS*, Δ*csrArsmEgacS* and Δ*rsmAcsrArsmEgacS* mutants in liquid broth at 25°C. At different time points, the optical density of the cell cultures was measured spectrophotometrically (600 nm). Mean values for four biological replicates are given; the error bars represent the standard errors of the mean.

Potential targets of the RsmA/RsmE repressor proteins in *P. fluorescens* SS101

To determine the potential targets of the RsmA and RsmE repressor proteins, we conducted a whole genome search for putative Rsm binding sites at or near the 5' untranslated leader mRNA by using the conserved motif 5'-$^{A}/_{U}$ CANGGANG$^{U}/_{A}$-3' (N is any nucleotide) (Lapouge

et al., 2008). A total of 17 genes were found with this conserved motif located in the ribosome binding site (RBS) (Table 2). For 6 of these 17 genes, transcription was significantly down-regulated in the *gacS*/*gacA* mutants and also in the *rsmYZ* double mutant (Table 2). These 6 genes included: PflSS101_0554 with unknown function; *gcd* (PflSS101_1096) encoding the quinoprotein glucose dehydrogenase; *ompA* (PflSS101_1239); *aprA* (PflSS101_2560), which encodes an extracellular protease; PflSS101_2598, a gene predicted to encode a formyl-transferase domain/enoyl-CoA hydratase/isomerase family protein; and *massAR* (PflSS101_3396), the LuxR-type transcriptional regulatory gene located upstream of the *massA* biosynthesis gene and essential for massetolide biosynthesis (de Bruijn & Raaijmakers, 2009a, de Bruijn & Raaijmakers, 2009b). There was no GacA box sequence upstream of *massA*, *massBC* or *massBC*R (LuxR type regulator downstream of *massBC*). Alignment of the 5' untranslated leader regions of these 6 putative target genes, with *hcnA* and *aprA* of *P. protegens* CHA0 and *P. aeruginosa* PAO1 as references, revealed the position of the consensus motif close to the RBS (Figure 4A). When the alignment for *massAR* was performed with genes of several closely related LuxR-type transcriptional regulator genes flanking other lipopeptide biosynthesis genes in different *Pseudomonas* species and strains, similar consensus motifs were found (Figure 4B). Based on these findings, we postulate that 1) the LuxR-type transcriptional regulator MassAR is the most likely target of the RsmA and RsmE repressor proteins in Gac/Rsm-mediated regulation of massetolide biosynthesis in *P. fluorescens* SS101, and 2) lipopeptide biosynthesis in other *Pseudomonas* species is most likely regulated in a similar manner.

Table 2. Predicted target genes of the RsmA and RsmE repressor proteins in *Pseudomonas fluorescens* SS101.

Gene locus	Gene descriptions	Fold change Δ*gacS*/wt[a]	P value	Fold change Δ*gacA*/wt[a]	P value	Fold change Δ*rsmYZ*/wt[a]	P value
PflSS101_0554	conserved hypothetical protein	-4.84	0.000926	-4.32	0.00118	-4.59	0.00108
PflSS101_0590	leucine rich repeat domain protein	1.04	0.389	1.12	0.00821	1.099	0.038
PflSS101_1073	conserved hypothetical protein	1.45	0.003	1.26	0.0125	1.389	0.00789
PflSS101_1096	quinoprotein glucose dehydrogenase (gcd)	-4.45	0.0000343	-4.32	0.0000232	-3.799	0.0000621
PflSS101_1198	putative pyocin R, lytic enzyme	-1.71	0.0326	-1.69	0.0332	-1.79	0.0272
PflSS101_1239	OmpA family lipoprotein	-22.68	2.05E-06	-16.24	4.42E-06	-11.77	3.45E-07
PflSS101_1789	putative membrane protein, PF05661 family	-1.28	0.0975	-1.27	0.0964	-1.25	0.104
PflSS101_2560	extracellular alkaline metalloprotease AprA	-44.57	0.0000135	-32.98	0.000109	-51.67	3.53E-07

PflSS101_2598	formyl transferase domain/enoyl-CoA hydratase/isomerase family protein	-37.92	1.04E-06	-32.81	9.01E-06	-35.88	8.84E-07
PflSS101_2670	UTP--glucose-1-phosphate uridylyltransferase	-1.06	0.441	1.17	0.0143	1.41	0.0021
PflSS101_2760	conserved hypothetical protein	1.23	0.183	1.33	0.0861	1.15	0.334
PflSS101_2801	hypothetical protein	-1.16	0.722	-1.01	0.988	1.07	0.87
PflSS101_3147	TonB-dependent outermembrane receptor	-1.09	0.00398	-1.02	0.721	1.02	0.732
PflSS101_3396	transcriptional regulator, MassAR	-43.6	5.76E-07	-36.27	0.0000314	-25.96	3.42E-06
PflSS101_3799	RmuC domain protein	1.22	0.169	1.17	0.254	1.11	0.469
PflSS101_4067	L-arabinose ABC transporter, ATP-binding proteinAraG	1.38	0.0521	1.7	0.0113	1.49	0.0676
PflSS101_5435	conserved hypothetical protein	1.09	0.669	-1.1	0.576	-1.11	0.562

All predicted target genes of Gac/Rsm cascade in *P. fluorescens* SS101 are indicated.
a. Positive values correspond to higher expression, negative values to lower expression (compared to the wild type). The target genes for which the expression is statistically significant (Fold change>=2; P< 0.001) in both the Δ*gacS, ΔgacA* and Δ*rsmYZ* mutant vs. wild type SS101 are shaded in grey.

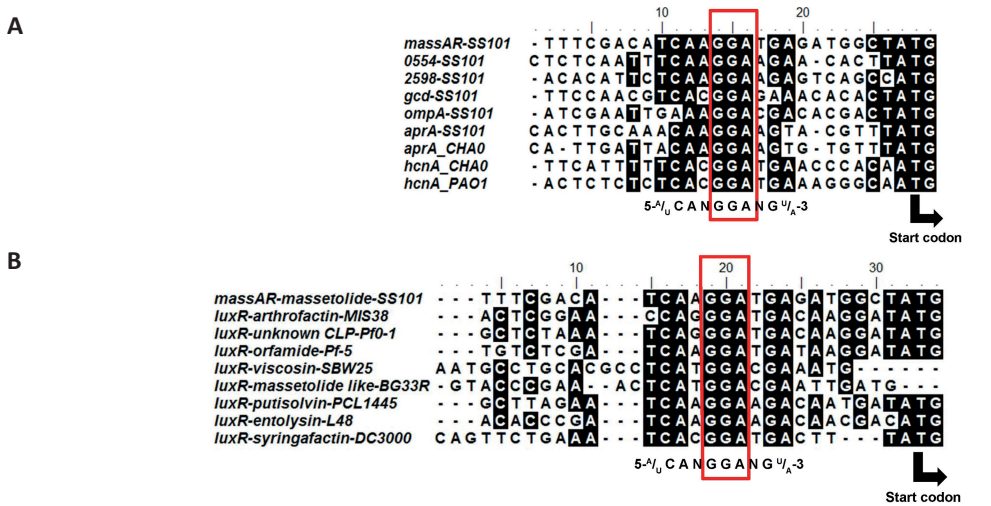

Figure 4. (A) Alignment of the upstream regions of five putative target genes of the RsmA and RsmE repressor proteins of *Pseudomonas fluorescens* SS101. The *aprA* and *hcnA* genes of *P. protegens* CHA0 and *P. aeruginosa* PAO1 were used as references. The translation initiation ATG codon is shown at the 3' end. (B). Alignment of the regions upstream of LuxR-type transcriptional regulatory genes that flank different lipopeptide biosynthesis gene clusters in *Pseudomonas fluorescens* SS101, *Pseudomonas* sp. MIS38, *P. fluorescens* Pf0-1, *P. protegens* Pf-5, *P. fluorescens* SBW25, *P. synxantha* BG33R, *P. putida* PCL1445, *P. entomophila* L48 and *Pseudomonas syringae* pv. *tomato* DC3000. The translation initiation ATG codon is shown at the 3' end.

Other Genes of the Rsm Regulon in *Pseudomonas fluorescens* SS101

To explore the potential roles of *rsmY* and *rsmZ* in global gene regulation in strain SS101, we conducted a genome-wide microarray analysis on the *rsmYZ* double mutant and the wild type strain, both sampled in the mid-exponential growth phase (OD_{600} ~ 0.6). In *rsmYZ,* the expression of *rsmY* and *rsmZ* was reduced 89 and 82-fold, respectively, due to the deletion of the corresponding genes. Various other significant changes in gene expression were observed with 121 and 272 genes significantly (FC>2.0; P<0.001) up- and down-regulated, respectively (Table S4; Table S5). Next to the genes involved in massetolide biosynthesis, the chitinase encoding gene *chiC* (PflSS101_3606) and a gene predicted to encode a bacterioferritin family protein (PflSS101_0584) were significantly down-regulated in the *rsmYZ* mutant. Moreover, 19 genes (PflSS101_5338-5358) homologous to the HSI-I type VI secretion system of *P. aeruginosa* (Mougous *et al.*, 2006) were down-regulated (Figure 5A). Another type VI secretion system HSI-II was not differentially regulated in the *rsmYZ* mutant. The putative functions of these type VI secretion systems in SS101, including a role in antibacterial activity or in plant-growth promotion (Decoin *et al.*, 2014), are yet unknown.

Transcriptomic analysis also revealed that *rebB_1* (PflSS101_0205) and *rebB_2* (PflSS101_0206) were down-regulated more than 44-fold and 93-fold, respectively, in the *rsmYZ* mutant. (Table S3). For certain endosymbionts, such as *Caedibacter* in *Paramecium,* these genes have been reported to encode insoluble proteins referred to as refractile bodies (R bodies) (Schrallhammer *et al.*, 2012). It has been noted that R bodies unwind under certain conditions and are associated with toxicity, i.e. the ability to kill symbiont-free competitors. For free living bacteria, including *P. fluorescens* SS101, the functions of these R bodies are not known yet. Given that not all down-regulated genes in *rsmYZ* double mutant harbor the conserved motif 5'-$^{A}/_{U}$ CANGGANG$^{U}/_{A}$-3' in the ribosome binding site (data not shown), we postulate that the altered expression of these genes might be due to indirect regulation by the Rsm regulon as was reported for *Pseudomonas aeruginosa* (Brencic & Lory, 2009).

Genes upregulated in the *rsmYZ* mutant represent genes involved in iron acquisition, chemotaxis and cell motility (Figure 5B). Also, *gabT* (PflSS101_0208), which is involved in γ-aminobutyric acid (GABA) utilization, was up-regulated in the *rsmYZ* mutant. Upregulation was also found for three genes of the *fagA-fumC-orfX-sodA* operon (PflSS101_0896, 0898, 0899) (Figure 5B), which functions in oxidative stress adaptation in *P. aeruginosa* (Polack *et al.*, 1996, Hassett *et al.*, 1997a, Hassett *et al.*, 1997b).

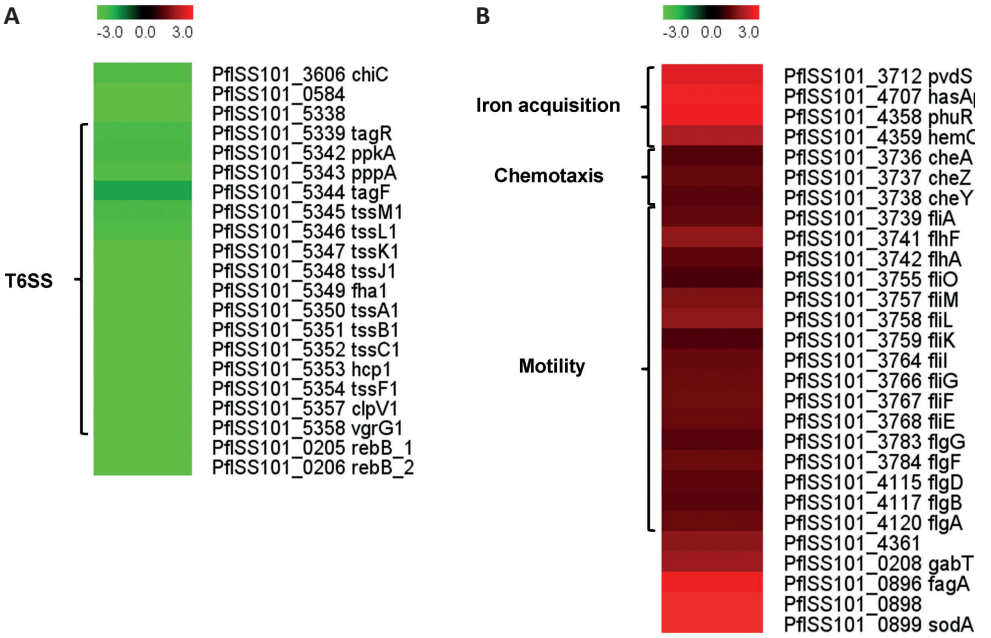

Figure 5. Whole genome transcriptome analysis of *Pseudomonas fluorescens* SS101 and the Δ*rsmYZ* mutant. Heatmaps showing significant $\log_2$-fold changes (P<0.001) in the expression of genes in the Δ*rsmYZ* vs. wild type cells. Wild type SS101 and the Δ*rsmYZ* mutant were grown in liquid KB at 25ºC to an optical cell density of OD_{600} = 0.6. The fold changes shown here represent averages of three biological replicates. Panel A represents known genes that were down-regulated in the Δ*rsmYZ* mutant, whereas panel B represents known genes up-regulated in the Δ*rsmYZ* mutant vs. wild type SS101. For a list of all genes differentially regulated in the Δ*rsmYZ* mutant vs. wild type SS101, we refer to Supplementary Tables S2 and S3.

Comparison of the Rsm regulon and the Gac regulon of *P. fluorescens* SS101

Many of the genes differentially regulated in the *rsmYZ* mutant of strain SS101 have also been reported previously to be differentially expressed in Gac mutants of other *Pseudomonas* species and strains (Hassan *et al.*, 2010, Cheng *et al.*, 2013, Brencic et al., 2009, Wang *et al.*, 2013). In *P. aeruginosa*, the GacS/GacA transduction system acts exclusively through its control over the transcription of *rsmY* and *rsmZ* (Brencic et al., 2009). However, the possibility that the system directly regulates other genes cannot be excluded for other *Pseudomonas* species and strains. For instance, in *L. pneumophila*, LetA (ortholog of GacS) regulates expression of flagellar genes by a mechanism that appears to be independent of RsmY and RsmZ (Sahr et al., 2009). In our study, comparative analyses of the Gac regulon and Rsm regulon of *P. fluorescens* SS101 were conducted according to Sahr et al. (2009). Briefly, we made a direct comparison (fold change >2.0, P value <0.05) of the gene expression pattern of Δ*gacA* and Δ*rsmYZ*. Additionally, we analysed genes differentially expressed in either Δ*gacA/wt* or in Δ*rsmYZ/wt.* Collectively, these analyses resulted in 5 genes differentially expressed in the Δ*gacA* mutant and 11 genes differentially expressed in the Δ*rsmYZ* mutant. One of

the 5 genes (PflSS101_2039) that was differentially expressed in the Δ*gacA* mutant is located directly downstream of *gacA.* Hence, its differential expression is most likely due to a polar effect of the *gac* mutation. Therefore, this gene was excluded from the comparison. In summary, the expression of 4 and 11 genes varied in Δ*gacA* and Δ*rsmYZ* mutants, respectively. One of these four genes is related to iron uptake, one is involved in amino acid transport and metabolism, and two genes are predicted to encode a hypothetical protein. The 11 genes uniquely expressed in the *rsmYZ* mutant (Table S6) were all significantly upregulated. One gene, encoding a secondary thiamine-phosphate synthase enzyme, showed the most increased expression (9-fold change) but its function in strain SS101 is not known yet. In summary, this analysis suggests that most, not all, of the genes controlled by GacS/GacA two-component system are controlled via RsmY/RsmZ.

Conclusions

Through *in silico* analyses of the genome of the rhizobacterium *P. fluorescens* SS101, 16 small RNAs were identified. Subsequent experiments revealed, for the first time, that the Rsm signal transduction pathway plays a critical role in the regulation of massetolide biosynthesis, a cyclic lipopeptide important for biofilm formation, swarming motility, antimicrobial activity and induction of systemic resistance in plants. We showed that the effects of the two sRNAs RsmY and RsmZ are channeled through the RsmA and RsmE repressor proteins and we predicted that the LuxR-type transcriptional regulator MassAR is one of the targets of these repressor proteins in strain SS101. To date, most information on the Rsm regulon in *Pseudomonas* species comes from studies on *P. aeruginosa* and *P. protegens*. Here, new information is provided that the Rsm system regulates lipopeptide biosynthesis in *P. fluorescens* SS101 and possibly other *Pseudomonas* species. Our study also provided, for the first time, a whole genome comparison of the Rsm and Gac regulons in a *Pseudomonas* species other than *P. aeruginosa*. The results of these analyses revealed that most, but not all of the genes controlled by RsmY/RsmZ are also controlled by the GacS/GacA two-component system, whereas in *P. aeruginosa* the Gac regulon controls downstream genes exclusively through the sRNAs RsmY and RsmZ.

Experimental procedures

Bioinformatic prediction of sRNAs in *Pseudomonas fluorescens* SS101 genome

sRNA searches were performed by BLAST and YASS (Noe & Kucherov, 2005) against the Rfam database (http://rfam.janelia.org/), as well as by ERPIN (Gautheret & Lambert, 2001), INFERNAL (Nawrocki *et al.*, 2009) and DARN (Zytnicki *et al.*, 2008), which are included in the RNAspace package (Cros *et al.*, 2011).

Bacterial strains and cultural conditions

Bacterial strains used in this study are listed in Table 3. *Pseudomonas fluorescens* strains were cultured in liquid King's medium B (KB) (King *et al.*, 1954) at 25°C. The *gacS* and *gacA* plasposon mutants were obtained with plasmid pTnModOKm (Dennis & Zylstra, 1998). *Escherichia coli* strain DH5α was used as a host for the plasmids used for site-directed mutagenesis. *E. coli* strains were grown on Luria-Bertani (LB) plates or in LB broth (Bertani, 1951) amended with the appropriate antibiotics.

Table 3. Bacterial strains and mutants used in this study

Strain	Relative characteristics	Reference source
Pseudomonas fluorescens		
SS101	Wild type, Rif^r	de Souza et al., 2003
Δ***gacS***	Plasposon mutant, Km^r	This study
Δ*gacA*	Plasposon mutant, Km^r	This study
Δ*rsmY*	*rsmY* deletion mutant	This study
Δ*rsmZ*	*rsmZ* deletion mutant	This study
Δ*rsmYZ*	*rsmY rsmZ* deletion mutant	This study
Δ*rsmAgacS*	*rsmA* deletion mutant in the Δ*gacS* background	This study
Δ*csrAgacS*	*csrA* deletion mutant in the Δ*gacS* background	This study
Δ*rsmEgacS*	*rsmE* deletion mutant in the Δ*gacS* background	This study
Δ*rsmAcsrAgacS*	*rsmA csrA* deletion mutant in the Δ*gacS* background	This study
Δ*rsmArsmEgacS*	*rsmA rsmE* deletion mutant in the Δ*gacS* background	This study
Δ*csrArsmEgacS*	*csrA rsmE* deletion mutant in the Δ*gacS* background	This study
Δ*rsmAcsrArsmEgacS*	*rsmA csrA rsmE* deletion mutant in the Δ*gacS* background	This study

1 Rif^r: Rifampin resistance; Km^r: Kanamycin resistance

Bacterial mutagenesis

Site-directed mutagenesis of the two small RNAs and three repressor protein genes was performed with the pEX18Tc suicide vector as described by de Bruijn et al (de Bruijn et al., 2008). The primers used are listed in Table S7. For each mutant construct, two fragments were amplified: Up and Down fragments. In the first-round PCR, the Up and Down fragments were amplified respectively. The first round PCR was performed with Pfu polymerase (Promega). The program used for the PCR consisting 1 min denaturation at 95°C, followed by 30 cycles of 95°C 1 min, Tm 30s, and 72°C 2 mins. The last step of the PCR was 72°C for 7 min. All fragments were separated on a 1% (wt/vol) agarose gel and purified with an Illustra GFX PCR DNA and Gel Band Purification Kit. The second round PCR was performed by mixing equimolar amounts of the up and down fragments as templates, up forward and down reverse primers were added in the Pfu PCR reaction system. All fragments were separated on a 1% agarose gel, and bands of the right size were purified with a Qiagen kit. The fragments were digested with EcoRI and HindIII and cloned into pEX18Tc. *E.coli* DH5α was transformed with pEX18TC-*rsmY*, pEX18TC-*rsmZ*, pEX18TC-*rsmA*, pEX18TC-*csrA* or pEX18TC-*rsmE* plasmids by heat shock transformation according to method of Inoue et al (Inoue *et al.*, 1990), and transformed colonies were selected on LB supplemented with 25 μg/ml tetracycline (Sigma). Integration of the

inserts was verified by restriction analysis of the plasmids. The plasmid inserts were verified by sequencing (Macrogen, Amsterdam, the Netherlands). The correct pEX18Tc-*rsmY* and pEX18Tc-*rsmZ* constructs were subsequently electroporated into *P. fluorescens* SS101; pEX18Tc-*rsmA*, pEX18Tc-*csrA* and pEX18Tc-*rsmE* constructs were transformed into the Δ*gacS* mutant. Electrocompetent cells were obtained according to the method of Choi et al. (2006), and electroporation occurred at 2.4 kV and 200 µF. After incubation in SOC medium (2% Bacto tryptone [Difco], 0.5% Bacto yeast extract [Difco], 10 mM NaCl, 2.5 mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose [pH 7]) for 2 h at 25°C, the cells were plated on KB supplemented with tetracycline (25µg/ml) and rifampin (50µg/ml). The single crossover colonies obtained were grown in LB overnight at 25°C and plated on LB supplemented 5% sucrose to accomplish the double crossover. The plates were incubated at 25°C for at least 48 h, and colonies were re-streaked on LB supplemented with tetracycline (25µg/ml) and on LB supplemented with 5% sucrose. Colonies that grew on LB with sucrose, but not on LB with tetracycline, were selected and subjected to colony PCR to confirm the deletion of the genes.

Lipopeptide extraction and RP-HPLC separation

Massetolide extractions and RP-HPLC analysis were conducted according to the methods described previously (de Bruijn et al., 2008, de Bruijn & Raaijmakers, 2009b). Briefly, *Pseudomonas* strains were grown on Pseudomonas agar plates (Pseudomonas agar 38g/L, glycerol 10g/L) for 48 h at 25°C. The cells were suspended in sterile de-mineralized water (~40 ml per plate), transferred to 50 mL tubes, shaken vigorously for 2 min and then centrifuged (30 min, 6000 rpm, 4°C). The culture supernatant was transferred to a new tube and acidified to pH 2.0 with 9% HCl. The precipitate was obtained by centrifugation (30 min, 6000 rpm, 4°C) and washed three times with acidified dH_2O (pH 2.0). The precipitate was resuspended in 5mL dH_2O and the pH adjusted to 8.0 with 0.2 M NaOH; the precipitate dissolves. The solution was centrifuged (30 min, 6000 rpm, 4°C) and the supernatant transferred to a new tube and subjected to lyophilization. Analytical high-pressure liquid chromatography (HPLC) separations were carried out on 5-µm C18 column (Waters Symmetry column, Waters, Etten-Leur, Netherlands), a 55-min linear gradient of 0 to 100% acetonitrile + 0.1% (v/v) trifluoroacetic acid (TFA) with a flow rate of 0.5ml/min. Detection was performed with a photodiode array detector (Waters) at wavelengths from 200 to 450 nm.

Swarming motility

Swarming motility assays of the bacterial strains and mutants were conducted according to the method described previously (de Bruijn & Raaijmakers, 2009b). Swarming motility of wild type strain SS101 and the mutants was assessed on soft [0.6% wt/vol] standard succinate agar medium (SSM) consisting of 32.8 mM K_2HPO_4, 22 mM KH_2PO_4, 7.6 mM $(NH_4)_2SO_4$, 0.8 mM $MgSO_4$, and 34 mM succinic acid and adjusted to pH 7 with NaOH. After autoclaving, the medium was cooled down in a water bath to 55°C and kept at 55°C for 1 h. Twenty ml of SSM was pipetted into a 9-cm-diameter petri dish, and the

plates were kept for 24 h at room temperature (20°C) prior to the swarming assay. For all swarming assays, the same conditions (agar temperature & volume, time period of storage of the poured plates) were kept constant to maximize reproducibility. Overnight cultures of wild type SS101, mutants, were washed three times with 0.9% NaCl, and 5 µL of the washed cell suspension ($1X10^{10}$ cells/ml) was spot inoculated in the centre of the soft SSM agar plate and incubated for 48-72 h at 25°C.

Transcriptional profiling

Wild type SS101,the Δ*gacA* and the Δ*rsmYZ* mutant were grown in King's medium B in 24-well plates, and harvested for RNA isolation at the mid-exponential growth stage (OD600 = 0.6). Cells of these strains were collected in triplicates. Total RNA was extracted with Trizol reagent (Invitrogen) and further purified with the NucleoSpin RNA kit (Macherey-Nagel). A tiling microarray for *Pseudomonas fluorescens* SS101 was developed in the MicroArray Department (MAD), University of Amsterdam (UvA), Amsterdam, the Netherlands. In total, 134,276 probes (60-mer) were designed with, in general, a gap of 32 nucleotides between adjacent probes on the same strand and an overlap by 14 nucleotides when regarding both strands. In addition, 5,000 custom negative control probes were hybridized, and used as an internal control to validate the designed probes in a comparative genomic hybridization (CGH) experiment of 4 arrays. Probes were annotated and assembled into probe sets for known genes based on location information retrieved from the Pathosystems Resource Integration Center (PATRIC, http://patricbrc.org). Probes outside of known genes were labeled as InterGenic Region (IGR). cDNA labelling was conducted as described previously (52). Briefly, cDNA was synthesized in presence of Cy3-dUTP (Cy3) for the test samples and with Cy5-dUTP (Cy5) for the common reference. The common reference was made by an equimolar pool of the test samples (3 µg per sample)**.** Five µg of total RNA per reaction was used and yielded 1.5-2.5 µg cDNA for each sample with more than 16 pmol of Cy3 or Cy5 dye per microgram. Hybridizations were performed according to Pennings et al. (Pennings *et al.*, 2011). Slides were washed according to the procedures described in the Nimblegen Arrays User's Guide - Gene Expression Arrays Version 5.0 and scanned in an ozone-free room with a Agilent DNA microarray scanner G2565CA (Agilent Technologies). Feature extraction was performed with NimbleScan v2.5 (Roche Nimblegen). Data pre-processing consisted of $\log_2$-transformation of the raw probe-intensity data, followed by a within slide Lowess normalization. Thus normalized sample (Cy3) channel intensities were summarized into probe sets values and normalized between arrays using the RMA (Robust Multi-Array Analysis) algorithm (Irizarry *et al.*, 2003). All results described were found to be significant using a false discovery rate of less than 5%. The Arraystar 12 software (DNASTAR, Madison, Wisconsin, USA) was used for analysing the pre-normalized array data. Statistical analyses were carried out with the normalized data using a moderated t-test to determine differential transcript abundance. Genes with a fold change > 2 and p-value < 0.05 were considered to be differentially regulated.

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Supplementary data

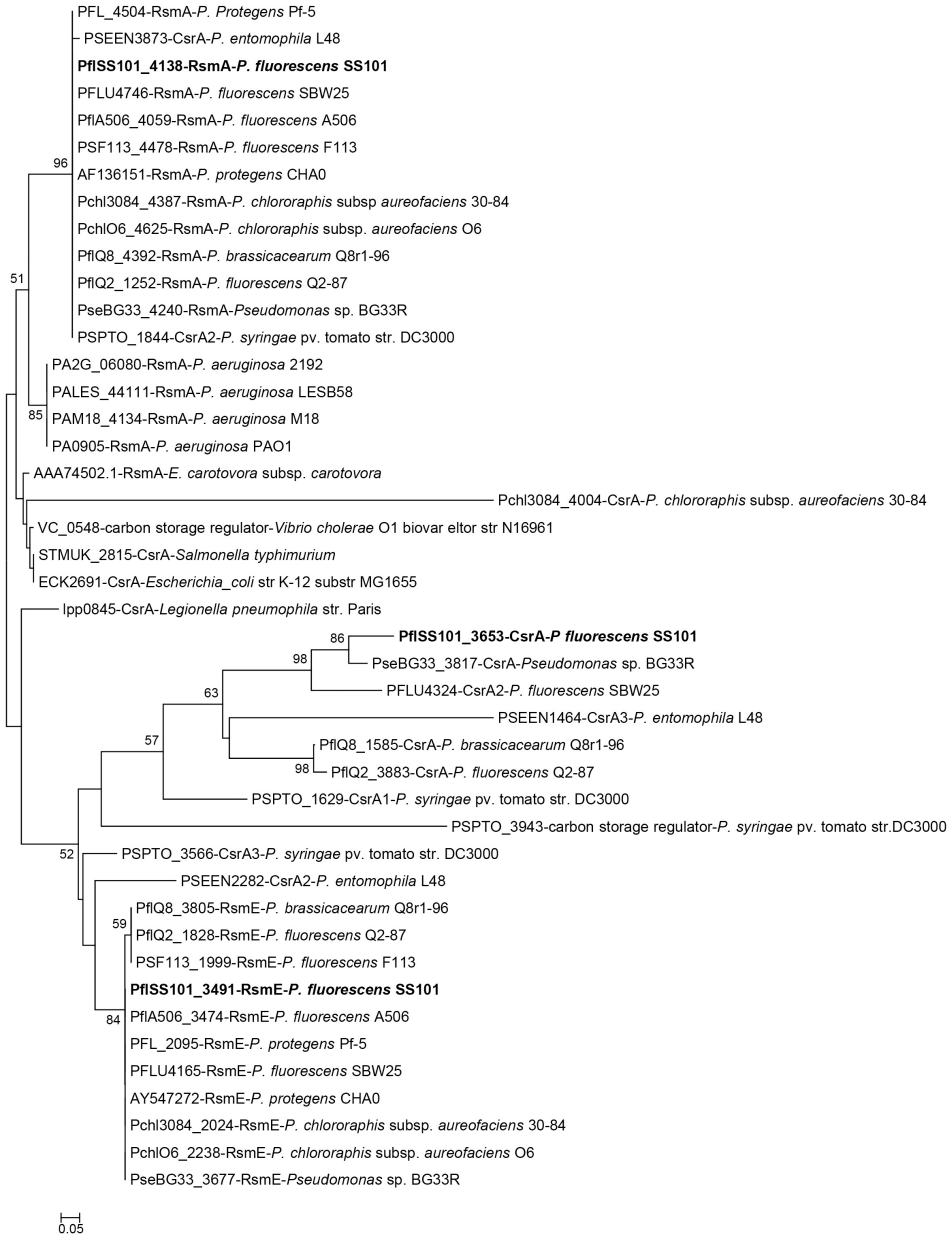
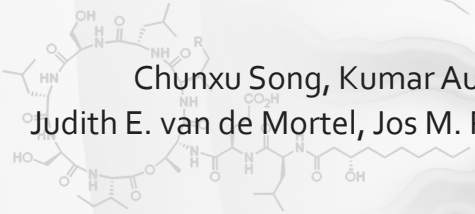


Figure S1. Phylogenetic analyses of RsmA/CsrA-like proteins in different *Pseudomonas* species and strains. The phylogenetic tree is based on amino acid sequences of RsmA, RsmE and CsrA from twenty-three bacterial genomes, and was generated by Neighbor-joining (NJ) (Saitou and Nei, 1987) in MEGA 6 (Tamura et al., 2013). The evolutionary distances were computed using Jones-Taylor-Thornton (JTT) model. The variation rate among sites was modelled with a gamma distribution. Bootstrap values (1,000 repetitions) are shown on branches. Rsm proteins from *P. fluorescens* strain SS101 are indicated in bold.

Supplementary tables are available on the website:
http://onlinelibrary.wiley.com/doi/10.1111/1751-7915.12190/suppinfo

Chapter 3

Discovery of new regulatory genes of lipopeptide biosynthesis in *Pseudomonas fluorescens*



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Abstract

Pseudomonas fluorescens SS101 produces the cyclic lipopeptide massetolide with diverse functions in antimicrobial activity, motility and biofilm formation. To understand how massetolide biosynthesis is genetically regulated in SS101, approximately 8,000 random plasposon mutants were screened for reduced or loss of massetolide production. Out of a total of 58 putative mutants, 45 had a mutation in one of the three massetolide biosynthesis genes *massA*, *massB* or *massC*. For 5 mutants, the insertions were located in the known regulatory genes *gacS, gacA,* and *clpP*. For the remaining 8 mutants, insertions were located in *clpA*, encoding the ClpP chaperone, in *phgdh,* encoding D-3-phosphoglycerate dehydrogenase, in the heat shock protein encoding *dnaK*, or in the transmembrane regulatory gene *prtR*. Genetic, chemical and phenotypic analyses showed that *phgdh*, *dnaK* and *prtR* are indeed involved in the regulation of massetolide biosynthesis, most likely by transcriptional repression of the LuxR-type regulator genes *massAR* and *massBCR*. In addition to their role in massetolide biosynthesis, *dnaK* and *prtR* were found to affect siderophore and extracellular protease(s) production, respectively. The identification of new regulatory genes substantially extended insights into the signal transduction pathways of lipopeptide biosynthesis in *P. fluorescens* and into regulation of other traits that may contribute to its life-style in the rhizosphere.

Introduction

Lipopeptides (LP) are produced by diverse bacterial genera and are composed of a lipid tail linked to a short linear or cyclic oligopeptide (Raaijmakers *et al.*, 2010). LP are surface-active compounds, exhibit broad-spectrum antibiotic activities and have diverse natural functions in motility, biofilm formation and virulence (Raaijmakers *et al.*, 2006, Raaijmakers & Mazzola, 2012). The LP massetolide A was identified in *Pseudomonas fluorescens* SS101, a biocontrol strain isolated from the wheat rhizosphere (de Souza *et al.*, 2003, de Bruijn *et al.*, 2008), and has potent surfactant and broad-spectrum antimicrobial activities (Gerard *et al.*, 1997, van de Mortel *et al.*, 2009). LP biosynthesis is governed by large multimodular nonribosomal peptide synthetases (NRPS) and is well studied in *Pseudomonas* and *Bacillus* (Finking & Marahiel, 2004, Raaijmakers et al., 2006). In contrast, relatively little is known about the regulatory networks and the signal transduction pathways involved in LP biosynthesis. Among the global regulatory systems, two-component regulators play an important role in the regulation of LP biosynthesis (Raaijmakers et al., 2010). For example, the GacS/GacA two-component system in *Pseudomonas* functions as a master switch and a mutation in either one of the two genes results in loss of LP production (Kitten *et al.*, 1998, Koch *et al.*, 2002, Dubern *et al.*, 2005, de Bruijn *et al.*, 2007). Also in *Bacillus*, LP biosynthesis is regulated by a two-component system as was shown for ComA/ComP in surfactin biosynthesis (Sullivan, 1998). Also quorum sensing plays a role in the regulation of LP biosynthesis in some species and strains. For example, in *Bacillus* the cell-density dependent pheromone ComX and the phosphatase RapC are involved in surfactin biosynthesis (Duitman *et al.*, 2007). For *Pseudomonas fluorescens* 5064 and *Pseudomonas putida* PCL1445, *N*-acyl homoserine lactones (*N*-AHLs) regulate viscosin and putisolvin biosynthesis, respectively (Cui *et al.*, 2005, Dubern *et al.*, 2006). However, for various other *Pseudomonas* strains belonging to the same species, including *P. fluorescens* strain SS101, subject of this study, no evidence was found for a role of *N*-AHL-mediated regulation of LP biosynthesis (Dumenyo *et al.*, 1998, Kinscherf & Willis, 1999, Andersen *et al.*, 2003, de Bruijn et al., 2007, de Bruijn et al., 2008). This indicates that cell-density dependent regulation of LP biosynthesis can differ among species and among strains of the same species. In addition to these two global regulatory systems, LuxR-type transcriptional regulators that flank the LP biosynthesis genes have been shown to regulate syringomycin, syringopeptin, syringofactin, putisolvin, entolysin, viscosin and massetolide biosynthesis (Lu *et al.*, 2002, Wang *et al.*, 2006, Berti *et al.*, 2007, Dubern *et al.*, 2008, de Bruijn & Raaijmakers, 2009a, Vallet-Gely *et al.*, 2010). In *P. putida*, also DnaK was identified as an additional regulator of putisolvin biosynthesis (Dubern et al., 2005).

The study presented here focuses on identification of regulatory genes of massetolide biosynthesis in the beneficial rhizobacterium *P. fluorescens* SS101. Massetolides consist of a 9-amino-acid cyclic peptide moiety linked to a 3-hydroxydecanoic acid tail and belong to the viscosin LP group (Raaijmakers et al., 2006). Massetolide biosynthesis

is governed by three NRPS genes, designated *massA*, *massB*, and *massC,* flanked by two LuxR-type regulatory genes *massAR* and *massBCR* (de Bruijn et al., 2008, de Bruijn & Raaijmakers, 2009a). We previously identified the two-component system GacS/GacA and the serine protease ClpP as regulators of massetolide biosynthesis in strain SS101 (de Bruijn & Raaijmakers, 2009b). At the transcriptional level, ClpP-mediated regulation of massetolide biosynthesis appears to operate independently from the regulation by GacS/GacA (de Bruijn & Raaijmakers, 2009b). Based on these previous findings, a tentative regulation model was proposed where ClpP regulates, alone or together with a yet unknown chaperone other than ClpX, massetolide biosynthesis via degradation of putative transcriptional repressors of *massAR* and/or via modulation of the citric acid cycle and amino acid metabolism (de Bruijn & Raaijmakers, 2009b). The aims of this study were to i) perform a genome-wide search for new regulatory genes of massetolide biosynthesis in *P. fluorescens* SS101, ii) determine the role of these genes in the regulation of massetolide production, and iii) investigate the putative role of these regulatory genes in other phenotypic traits of *P. fluorescens* SS101. To this end, we screened two independent random plasposon mutant libraries (~8,000 mutants total) for a reduced or loss of massetolide production. Thirteen putative regulatory mutants were found. Genetic, phenotypic, chemical and transcriptional analyses were performed to elucidate the functions of three regulatory genes in massetolide biosynthesis and in other phenotypic traits, including swarming motility, siderophore production and extracellular protease activity.

Results

Screening for massetolide-deficient mutants of P. fluorescens SS101

Two independent libraries of 520 and 7,500 random Tn*Mod* plasposon mutants of strain SS101 were screened in a drop-collapse assay (Figure 1A) for reduced or loss of massetolide production. The drop collapse assay is a highly reliable proxy (de Bruijn et al., 2008, de Bruijn & Raaijmakers, 2009b) for massetolide production in *P. fluorescens* SS101. A total of 58 putative massetolide-deficient mutants were found. The regions flanking the plasposon insertion were sequenced for all 58 mutants. In 45 mutants, the insertion was located in *massA*, *massB*, or *massC*. The insertions in the other 13 mutants were located in three genes described previously for their role in massetolide biosynthesis (de Bruijn & Raaijmakers, 2009b) and in four putative new regulatory genes. The three known regulatory genes were the caseinolytic protease gene *clpP* (n=1), the sensor kinase gene *gacS* (n=3) and its cognate response regulator gene *gacA* (n=1). The four putative new regulatory genes were *clpA* (n=4; PflSS101_3193), *dnaK* (n=2; PflSS101_4633), *prtR* (n=1; PflSS101_3280), and *phgdh* (n=1; PflSS101_5176). The *clpA* gene encodes the chaperone of the ClpP serine protease and most likely regulates massetolide biosynthesis via ClpP. The regulatory role of ClpA and its interplay with ClpP was not further investigated here. Instead, a more detailed functional analysis was conducted for *phgdh, dnaK* and *prtR*.

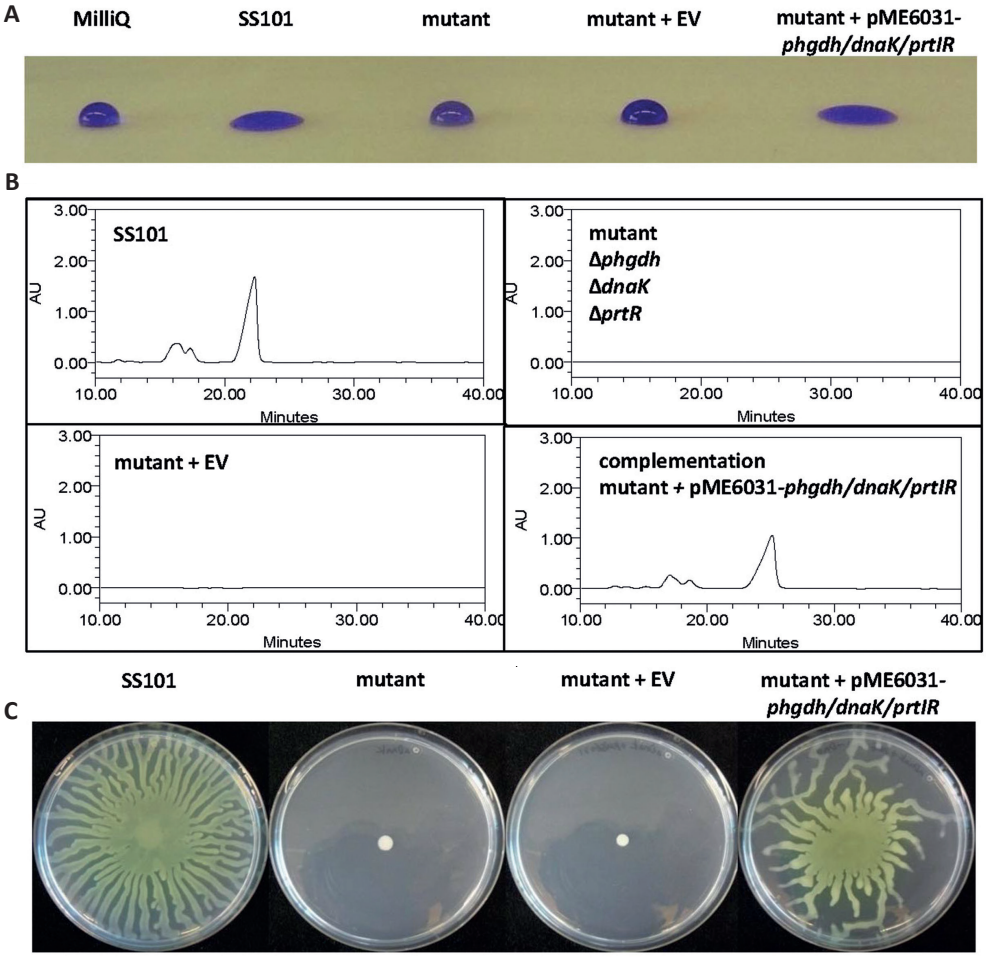

Figure 1. Phenotypic and chemical analyses of three massetolide-deficient mutants of *Pseudomonas fluorescens* strain SS101.

(A) Drop collapse assay with cell suspensions of wild-type SS101 and plasposon mutants disrupted in *phgdh, dnaK,* or *prtR*. Each of these three mutants was complemented with the corresponding gene and designated mutants+pME6031-*phgdh/dnaK/prtIR.* Mutant+EV represents each of the mutants with the plasmid pME6031 used for genetic complementation (empty-vector control). Bacterial cells grown for 2 days at 25°C on KB agar plates were suspended in sterile water to a final density of $1x10^{10}$ cells/ml and 10-µl droplets were spotted on parafilm; crystal violet was added to the droplets to facilitate visual assessment. A flat droplet is a highly reliable proxy for the production of the surface-active lipopeptide massetolide. One representative phenotype (Δ*dnaK*, Δ*dnaK*+EV and Δ*dnaK*+pME6031-*dnaK*) is shown.

(B) RP-HPLC chromatograms of cell-free culture extracts of wild-type SS101, the three plasposon mutants, mutants+EV (empty-vector control), and complemented mutants as described in panel A. The wild-type strain SS101 produces massetolide A (retention time of approximately 23-25 min) and various other derivatives of massetolide A (minor peaks with retention times ranging from 16 to 18 min) which differ from massetolide A in the amino acid composition of the peptide moiety. One representative chromatogram (Δ*dnaK*, Δ*dnaK*+EV and Δ*dnaK*+pME6031-*dnaK*) is shown.

(C) Swarming motility of wild-type SS101 and mutants on soft (0.6% [wt/vol]) agar plates. Five microliters ($1x10^{10}$ cells/ml) of washed overnight cultures of wild-type SS101 or mutants was spot inoculated in the center of a soft agar plate and incubated for 48 to 72 h at 25°C. One representative phenotype (Δ*dnaK*, Δ*dnaK*+EV and Δ*dnaK*+pME6031-*dnaK*) is shown.

To confirm the role of these three genes in the regulation of massetolide biosynthesis, RP-HPLC analysis showed that these three mutants were indeed all deficient in the production of massetolide A and its derivatives (Figure 1B). Complementation of the mutants with each of the corresponding target genes cloned into the stable vector pME6031 restored massetolide production, whereas no massetolides were detected in the empty-vector control (Figure 1A, 1B). Massetolide biosynthesis is known to be essential for swarming motility of strain SS101 (de Bruijn et al., 2008). All three mutants lost their ability to swarm on soft agar (0.6% w/v) (Figure 1C). Swarming motility was restored by complementation with the corresponding target gene, albeit with a slightly different swarming pattern (Figure 1C) which may be due to effects of the copy number of the plasmid used for complementation. Collectively, these results indicate that all three genes are indeed required for massetolide biosynthesis in SS101.

Characterization of regulatory mutant Δphgdh

D-3 phosphoglycerate dehydrogenase (*phgdh*) is known to be involved in the biosynthesis of the amino acid L-serine. It converts 3-phosphoglycerate into 3-phosphohydroxypyruvate which in turn is converted into 3-phosphoserine by 3-phosphoserine aminotransferase (PSAT). Finally, 3-phosphoserine is converted into L-serine by phosphoserine phosphatase (PSP) (van der Crabben *et al.*, 2013). In *P. fluorescens* SS101, the *phgdh* gene is 1230 bp and BlastX analysis showed 89-99% identity to homologues in other *Pseudomonas* genomes (Figure 2A). A mutation in *phgdh* significantly reduced the expression of *massA*, *massB*, and *massC,* and of the LuxR-type transcriptional regulators *massAR* and *massBCR* (Figure 2B). Growth of the *phgdh* mutant was adversely affected in the stationary phase and this deficiency was largely restored by genetic complementation (Figure 2C). Based on the drop collapse assay, which is a highly reliable proxy for massetolide production in *P. fluorescens* SS101, we observed a restoration of massetolide production to wild type level with the addition of increasing concentrations of L-serine to the growth medium (Figure 2D). Collectively, these results indicate that *phgdh* regulates massetolide biosynthesis via modulation of L-serine biosynthesis.

Characterization of regulatory mutant ΔdnaK

The *dnaK* gene codes for a molecular chaperone of the Hsp70 protein family and was shown previously to regulate, together with its adjacent genes *dnaJ* and *grpE,* the biosynthesis of the LP putisolvin in *P. putida* PCL1445 (Dubern et al., 2005). In *P. fluorescens* SS101, the complete *dnaK* gene comprised 1923 bp and BlastX analysis showed 86-97% identity to *dnaK* in other *Pseudomonas* genomes (Figure 3A). Like in *P. putida* PCL1445 and other *Pseudomonas* species, *dnaK* is flanked in strain SS101 by the chaperone encoding gene *dnaJ* and the heat shock protein encoding gene *grpE* (Figure 3A). qRT-PCR analyses showed that the transcript levels of *massA*, *massB*, *massC* and the two regulatory genes *massAR* and *massBCR* were significantly decreased in the *dnaK* mutant (Figure 3B). The growth rate of the *dnaK* mutant in KB broth was reduced relative to that of wild type SS101, particularly in the lag phase; the mutant also reached relatively lower cell densities in the stationary phase (Figure 3C).

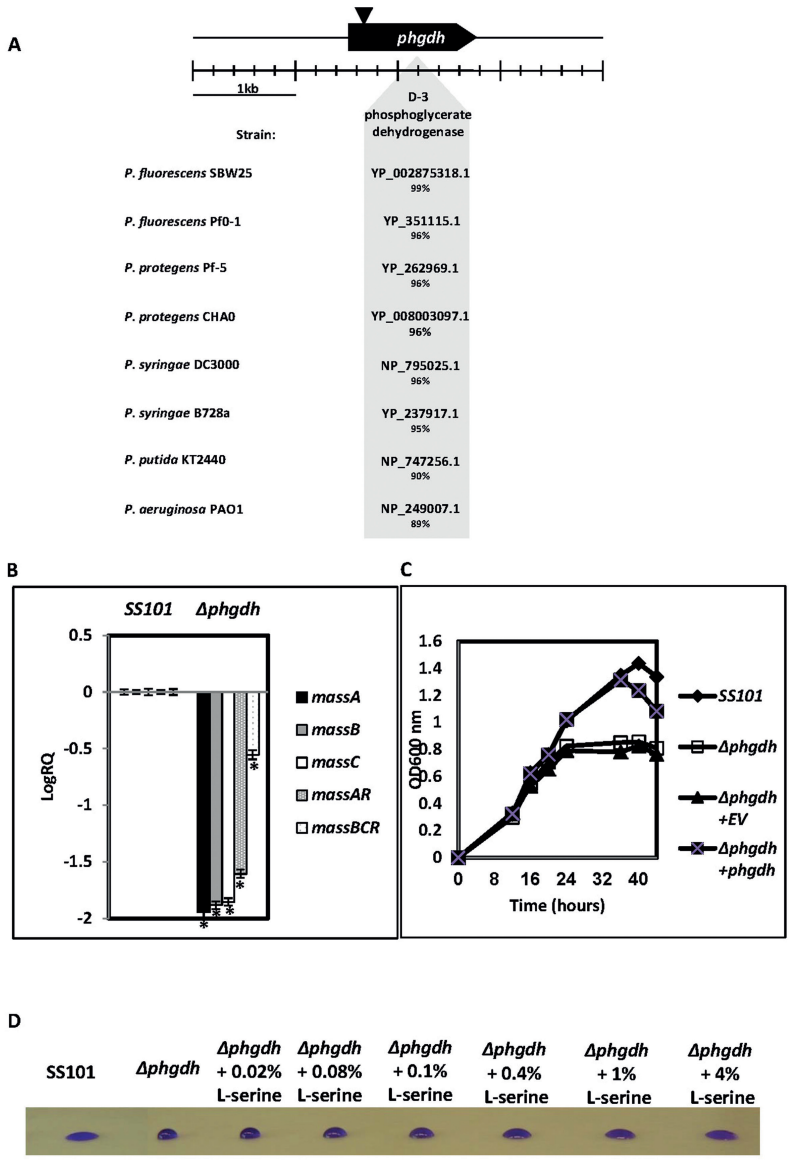

Figure 2. Characterization of the *phgdh* mutant of *Pseudomonas fluorescens* SS101.
(A) The *phgdh* gene (PflSS101_5176) in *P. fluorescens* SS101 and the percentages of amino acid identity with its corresponding homologues in other *Pseudomonas* species and strains. The triangle indicates the position of the plasposon insertion in the *phgdh* gene.
(B) Transcript levels of *massA*, *massB*, *massC, massAR* and *massBCR* in wild-type SS101 and the *phgdh* mutant. qRT-PCR analysis was performed on RNA extracted from cells of SS101 and *phgdh* mutant from the mid-exponential growth phase (OD_{600} = 0.6). The transcript level of each of the genes was corrected for the transcript level of the housekeeping gene *rpoD* [ΔC_T = C_T (gene *x*) - C_T(*rpoD*)] and is presented relative to the transcript levels in wild-type SS101 (log RQ), where RQ equals $2^{-[\Delta CT(mutant) - \Delta CT (wild\ type)]}$. Mean values of four biological replicates are given; the error bar represents the standard errors of the mean. The asterisk indicates a statistically significant (P<0.05) difference between the mutant and the wild-type SS101.
(C) Growth of wild-type SS101 and *phgdh* mutant, *phgdh* mutant+EV (empty-vector control), and *phgdh*

mutant+pME6031-*phgdh* at 25°C. At different time points, the optical density of the cell cultures was measured spectrophotometrically (OD_{600} nm). Mean values for four biological replicates are given; the error bars represent the standard error of the mean.
(D) Drop collapse assay with cell cultures of wild-type SS101 and *phgdh* mutant, *phgdh* mutant+EV (empty-vector control), and *phgdh* mutant+pME6031-*phgdh*. Bacteria were grown for 2 days at 25°C in KB broth supplemented with different concentrations of L-serine. Droplets (10-µl) of the cell-free culture supernatant were spotted on parafilm; crystal violet was added to the droplets to facilitate visual assessment. A flat droplet is a highly reliable proxy for massetolide production (de Bruijn *et al.*, 2008, de Bruijn & Raaijmakers, 2009a).

In *P. putida* PCL1445, putisolvin production decreased with increasing temperature (Dubern et al., 2005). Here we also observed that, based on tensiometric analysis of the cell-free culture filtrates, massetolide production decreased with increasing temperatures for both wild type SS101 and the complemented *dnaK* mutant (Figure S1). Next to these phenotypes, we observed a significant difference in fluorescence between wild type SS101 and the *dnaK* mutant when grown in King's medium B (KB) broth, with the wild type being more green-fluorescent than the *dnaK* mutant. Subsequent phenotypic analysis on CAS-agar indicator plates confirmed that a mutation in *dnaK* adversely affects siderophore production (Figure 3D). Spectrophotometric analysis (A_{400nm}) of cell-free culture filtrates of SS101 and of the *dnaK* mutant grown in KB broth confirmed the results of the CAS agar plate assays, with a reduced siderophore production in the *dnaK* mutant (not shown). This alteration in siderophore production by the *dnaK* mutant was observed at four different incubation temperatures (i.e. 10°C, 18°C, 25°C and 28°C; not shown) and therefore seems not to be temperature dependent which is in contrast with the results shown for massetolide production. Collectively, these results showed that, along with massetolide production, a mutation in *dnaK* also affects siderophore production in SS101.

Characterization of regulatory mutant ΔprtR

PrtR was previously reported to be a novel anti-sigma factor and transmembrane activator which interacts with ECF (extra-cytoplasmic function) sigma factors of the σ^{70} family (Burger *et al.*, 2000, Mascher, 2013). The neighbouring *prtI* gene is an ECF sigma factor and usually encoded in an operon with *prtR* (Mascher, 2013). BlastX analysis of the complete *prtR* gene (738 bp) of SS101 showed 43-87% identity to *prtR* homologues in *P. putida, P. fluorescens* and *P. protegens* genomes (Figure 4A). The *prtI* and *prtR* genes were not found in *P. syringae* or *P. aeruginosa* strains (Figure 4A). In SS101, *prtR* is flanked by *prtI* and one transcription unit was predicted based on SoftBerry FGENESB analysis (Figure 4A). qRT-PCR analyses showed that, similar to the results described above for the other two regulatory genes, transcript levels of the three massetolide biosynthesis genes and the two LuxR-type regulatory genes were significantly decreased in the *prtR* mutant (Figure 4B). The *prtR* mutation did not affect growth of *P. fluorescens* SS101 in the lag and exponential phase, but did so in the stationary phase (Figure 4C). Complementation of the *prtR* mutation with the *prtIR* operon did not fully restore the growth, which may be due to effects of plasmid-mediated copy number of *prtR*, *prtI* or both.

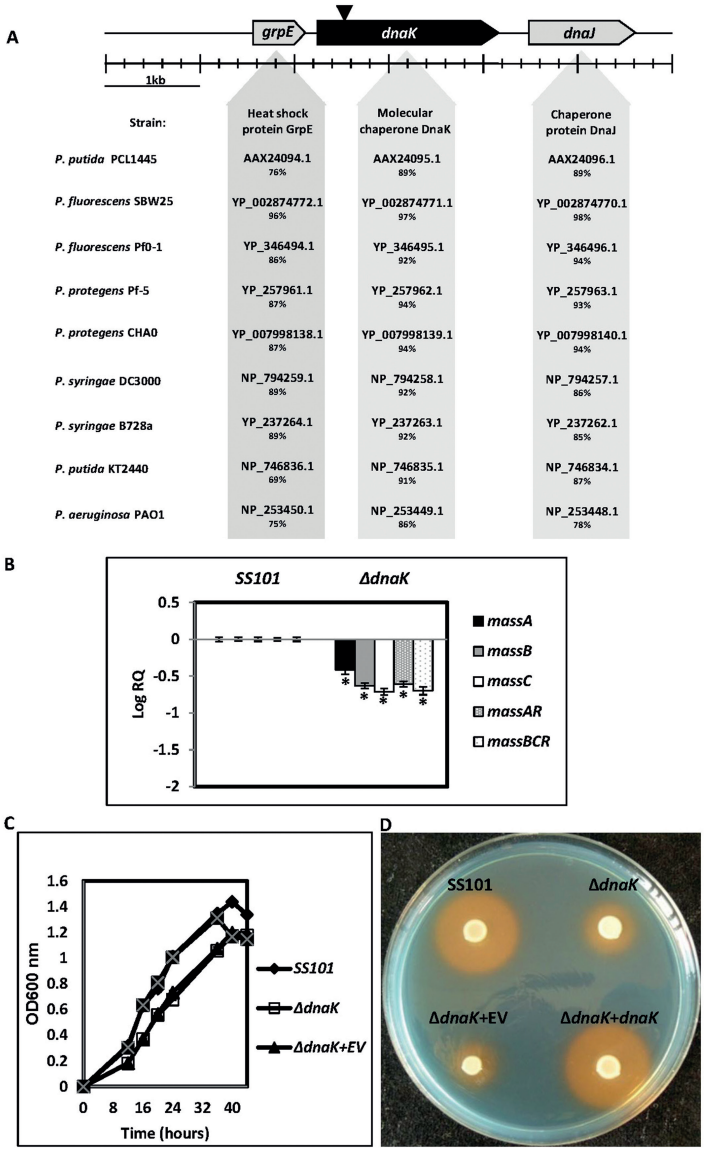

Figure 3. Characterization of the *dnaK* mutant of *Pseudomonas fluorescens* SS101

(A) The *dnaK* gene (PflSS101_4633) and flanking genes *grpE*, *dnaJ* in *P. fluorescens* SS101 and the percentages of amino acid identity with their corresponding homologues in other *Pseudomonas* species and strains. The triangle indicates the position of the plasposon insertion in the *dnaK* gene.

(B) Transcript levels of *massA*, *massB*, *massC, massAR* and *massBCR* in wild-type SS101 and the *dnaK* mutant. qRT-PCR analysis was performed on RNA extracted from cells of SS101 and *dnaK* mutant from the mid-exponential growth phase (OD_{600} = 0.6). The transcript level of each of the genes was corrected for the transcript level of the housekeeping gene *rpoD* [$\Delta C_T = C_T$ (gene *x*) - C_T(*rpoD*)] and is presented relative to the transcript levels in wild-type SS101 (log RQ), where RQ equals $2^{-[\Delta CT(mutant) - \Delta CT (wild\ type)]}$. Mean values of four biological replicates are given; the error bar represents the standard errors of the mean. The asterisk indicates a statistically significant (P<0.05) difference between the mutant and the wild-type SS101.

(C) Growth of wild-type SS101 and *dnaK* mutant, *dnaK* mutant+EV (empty-vector control), and *dnaK*

mutant+pME6031-*dnaK* at 25°C. At different time points, the optical density of the cell cultures was measured spectrophotometrically (OD_{600} nm). Mean values for four biological replicates are given; the error bars represent the standard error of the mean.
(D) Siderophore production of wild-type SS101, *dnaK* mutant, *dnaK* mutant+EV (empty-vector control), and *dnaK* mutant+pME6031-*dnaK*. Five microliters (10^9 cells/mL) of washed overnight cultures of wild-type SS101 and mutant were spot-inoculated in the center of a CAS indicator agar plate and incubated for 48 to 96 h at 10°C, 18°C, 25°C, 28°C respectively. A halo is indicative of siderophore production. The results of plates incubated at 25°C are shown and are representative of the results obtained for the other incubation temperatures tested.

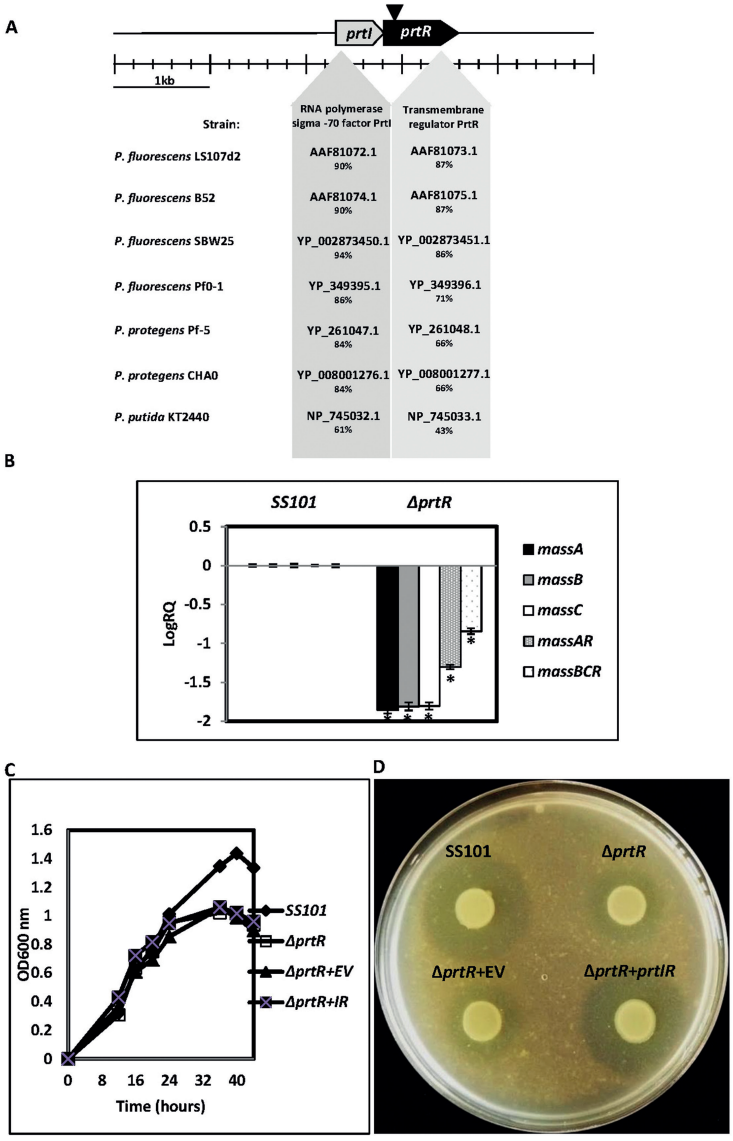

Figure 4. Characterization of the *prtR* mutant of *Pseudomonas fluorescens* SS101.
(A) The *prtR* gene (PflSS101_3280) and flanking gene *prtI* in *P. fluorescens* SS101 and the percentages of amino acid identity with their corresponding homologues in other *Pseudomonas* species and strains. The

triangle indicates the position of the plasposon insertion in the *prtR* gene.
(B) Transcript levels of *massA*, *massB*, *massC, massAR* and *massBCR* in wild-type SS101 and the *prtR* mutant. qRT-PCR analysis was performed on RNA extracted from cells of SS101 and *prtR* mutant from the mid-exponential growth phase (OD_{600} = 0.6). The transcript level of each of the genes was corrected for the transcript level of the housekeeping gene *rpoD* [$\Delta C_T = C_T$ (gene *x*) - C_T(*rpoD*)] and is presented relative to the transcript levels in wild-type SS101 (log RQ), where RQ equals $2^{-[\Delta CT(mutant) - \Delta CT (wild\ type)]}$. Mean values of four biological replicates are given; the error bar represents the standard errors of the mean. The asterisk indicates a statistically significant (P<0.05) difference between the mutant and the wild-type SS101.
(C) Growth of wild-type SS101 and *prtR* mutant, *prtR* mutaWnt+EV (empty-vector control), and *prtR* mutant+pME6031-*prtIR* at 25°C. At different time points, the optical density of the cell cultures was measured spectrophotometrically (OD_{600} nm). Mean values for four biological replicates are given; the error bars represent the standard error of the mean.
(D) Extracellular protease activity of wild-type SS101, *prtR* mutant, *prtR* mutant+EV (empty-vector control), and *prtR* mutant+pME6031-*prtIR*. Five microliters (10^9 cells/mL) of washed overnight cultures of wild-type SS101 and mutants were spot-inoculated in the center of a Skim Milk Agar (SMA) plate and incubated for 48 to 72 h at 25°C, 29ºC, 30ºC respectively. A halo is indicative of extracellular protease production. The results of plates incubated at 25°C are shown and are representative of the results obtained for the other incubation temperatures tested.

In *P. fluorescens* LS107d2, the *prtIR* genes were shown to be involved in temperature-dependent regulation of extracellular protease activity (Burger *et al.*, 2000). In the study by Burger *et al.* (2000), the *prtR* and *prtI* mutants produced extracellular proteases at 23ºC but not at 29ºC. In SS101, extracellular protease activity was reduced in the *prtR* mutant (Figure 4D) at all three temperatures (25ºC, 29ºC, 30ºC) tested. Collectively, these results indicate that *prtR* regulates massetolide biosynthesis as well as extracellular protease production in SS101.

Discussion

Massetolides are lipopeptide biosurfactants required for swarming motility, biofilm formation and broad-spectrum antimicrobial activities (Gerard et al., 1997, de Souza et al., 2003, de Bruijn et al., 2008, van de Mortel et al., 2009). Here, we analyzed 58 massetolide-deficient mutants and discovered four new regulatory genes of massetolide biosynthesis, i.e. *clpA*, *phgdh*, *dnaK* and *prtR*. Consistent with the results of our previous study on the role of the GacS/A two-component system and the ClpP serine protease (de Bruijn & Raaijmakers, 2009b), mutations in *phgdh, dnaK* and *prtR* adversely affected transcription of the three *massABC* biosynthesis genes, most likely through transcriptional repression of one or both of the LuxR-type regulatory genes *massAR* and *massBCR* (Figure 5). Although we screened a large library of more than 8,000 random mutants, we most likely did not cover the entire genome in strain SS101 to identify all regulatory genes of massetolide biosynthesis. Work by Liberati et al (2006) on 34,176 random mutants of *Pseudomonas aeruginosa* PA14 (genome 6.53 Mb), showed that 75% of the predicted genes were mapped with on average 4.3 transposon insertions per gene. They also pointed out that there is bias in transposon insertion sites and that large genes tend to have a higher frequency of insertions than relatively small genes. This may explain that the majority (n=45) of the massetolide mutants had the insertion

in the large (6-13 kb) *massABC* genes and that no mutants were found in, for example, the small *massAR* (795 bp) and *massBCR* (672 bp) genes.

Based on our previous study (de Bruijn & Raaijmakers, 2009b), we proposed a model in which the serine protease ClpP regulates massetolide biosynthesis, alone or together with a chaperone other than ClpX, by degradation of putative transcriptional repressors of *massAR* or via modulation of the citric acid cycle and amino acid metabolism (Figure 5). In that study, we also showed that addition of proline and glutamic acid to the growth medium can partially complement the deficiency in swarming motility of the *clpP* mutant (de Bruijn & Raaijmakers, 2009b). The results obtained here for *phgdh,* a key gene in L-serine biosynthesis, further extend the hypothesis that amino acid metabolism and in particular serine biosynthesis affects massetolide production. Given that serine makes up two out of the nine amino acids in massetolide A, a possible scenario may be that a *phgdh* mutation depletes the cellular pool of serine thereby affecting massetolide biosynthesis/assembly (Figure 5). More experiments will be required to support this hypothesis.

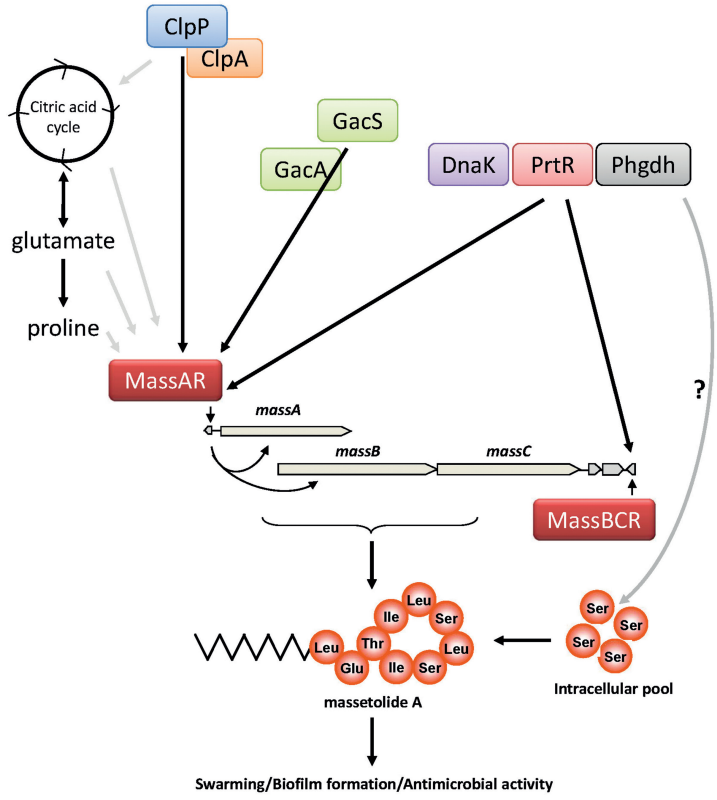

Figure 5. Proposed model for the genetic regulation of massetolide biosynthesis in *P.* fluorescens strain SS101. The darkly shaded arrows are based on experimental data obtained by De Bruijn & Raaijmakers (2009) and in this study; the lightly shaded arrows are hypothetical and not based on experimental data.

DnaK, together with the flanking genes *dnaJ* and *grpE,* was identified previously by Dubern *et al.* (2005) for its role in the regulation of putisolvin biosynthesis in *P. putida*. They postulated that DnaK, DnaJ and GrpE may be required for proper folding or activity of other regulators of the putisolvin biosynthesis gene *psoA* or alternatively may be necessary for proper assembly of the putisolvin NRPSs (Dubern et al., 2005). In our study, we found that DnaK regulates massetolide as well as siderophore biosynthesis in *P. fluorescens* SS101. Based on these results one might speculate that DnaK has a more global function in the regulation of NRPS genes, but more work needs to be done to support this suggestion. For *P. putida*, Dubern *et al.* (Dubern et al., 2005) also showed that *dnaK* expression was under the control of GacS/GacA. Preliminary results of whole-genome microarray analyses of the Gac mutant of strain SS101, however, suggest that *phgdh, dnaK*, *prtR* and *clpA* expressions are not under the control of the GacS/GacA system. Hence, in the adapted regulation model (Figure 5), the Gac-signal transduction route is kept separate from the other regulatory genes.

Bacteria possess different means to connect an extracellular input with an appropriate cellular response. Following one-component and two-component systems, extra-cytoplasmic function σ factors (ECFs) represent the third most abundant type of bacterial signal transduction. PrtR interacts with ECF sigma factors of the σ^{70} family and is required for *aprX* protease expression in *P. fluorescens* LS107d2 (Burger et al., 2000). In *P. entomophila*, *prtR* affects *aprA* protease production and contributes to pathogenicity (Liehl *et al.*, 2006). *PrtR* was also found in *P. fluorescens* WH6 to regulate the biosynthesis of the germination-arrest factor (GAF), which is a predicted small peptide or amino acid analog with herbicidal activity (Kimbrel *et al.*, 2010, Halgren *et al.*, 2013). Here, we showed, for the first time, that *prtR* also regulates massetolide biosynthesis in *P. fluorescens* SS101.

In conclusion, the identification of at least three new regulatory genes substantially extended our insight into the regulatory network of lipopeptide biosynthesis in *P. fluorescens* SS101. Based on the results presented we postulate that these three genes most likely regulate massetolide biosynthesis via one or both of the LuxR-type transcriptional regulators flanking the *massA* and *massBC* biosynthesis genes. Apart from their role in massetolide biosynthesis, we also showed that *dnaK* and *prtR* affect other traits in strain SS101 that may contribute to its life-style in the rhizosphere.

Materials and Methods

Bacterial strains and cultural conditions

Bacterial strains used in this study are listed in Table S1. *Pseudomonas fluorescens* strains were cultured in liquid King's medium B (KB) at 25°C. The *dnaK*, *prtR,* and *phgdh* plasposon mutants were obtained with plasmid pTnModOKm (Dennis & Zylstra, 1998). *Escherichia coli* strain DH5α was the host for the plasmids used for genetic

complementation. *E.coli* strains were grown on Luria-Bertani (LB) plates or in LB broth amended with the appropriate antibiotics.

Lipopeptide extraction and RP-HPLC separation

Massetolide extractions and RP-HPLC analysis were conducted according to the methods described previously (de Bruijn et al., 2008, de Bruijn & Raaijmakers, 2009b). Briefly, *Pseudomonas* strains were grown on Pseudomonas agar plates for 48 h at 25°C. The cell biomass was suspended in sterile de-mineralized water (~40 ml per plate), transferred to 50 mL tubes, shaken vigorously for 2 min and then centrifuged (30 min, 6000 rpm, 4°C). The culture supernatant was transferred to a new tube and acidified to pH 2.0 with 9% HCl. The precipitate was obtained by centrifugation (30 min, 6000 rpm, 4°C) and washed three times with acidified dH_2O (pH 2.0). The precipitate was resuspended in 5mL dH_2O and the pH adjusted to 8.0 with 0.2 M NaOH; the precipitate dissolves. The solution was centrifuged (30 min, 6000 rpm, 4°C) and the supernatant transferred to a new tube and subjected to lyophilization.

Swarming motility

Swarming motility assays of the bacterial strains and mutants were conducted according to the method described previously (de Bruijn & Raaijmakers, 2009b). Swarming motility of wild type strain SS101 and the mutants was assessed on soft [0.6% wt/vol] standard succinate agar medium (SSM) consisting of 32.8 mM K_2HPO_4, 22 mM KH_2PO_4, 7.6 mM $(NH_4)_2SO_4$, 0.8 mM $MgSO_4$, and 34 mM succinic acid and adjusted to pH 7 with NaOH. After autoclaving, the medium was cooled down in a water bath to 55°C and kept at 55°C for 1 h. Twenty ml of SSM was pipetted into a 9-cm-diameter petri dish, and the plates were kept for 24 h at room temperature (20°C) prior to the swarming assay. For all swarming assays, the same conditions (agar temperature & volume, time period of storage of the poured plates) were kept constant to maximize reproducibility. Overnight cultures of wild type SS101, mutants, were washed three times with 0.9% NaCl, and 5 µL of the washed cell suspension ($1X10^{10}$ cells/ml) was spot inoculated in the centre of the soft SSM agar plate and incubated for 48-72 h at 25°C.

Transcriptional analysis

The transcriptional analyses were conducted largely according to the method described previously (de Bruijn & Raaijmakers, 2009b), except that the SensiMixTM SYBR Kit was used for qRT-PCR instead of the SYBR Green Core kit (Eurogentee). In brief, RNA was extracted from the frozen bacterial cells with Trizol reagent (Invitrogen) and the Nucleospin kit. One µg RNA was used for cDNA synthesis with Superscript III (Invitrogen) according to the manufacturer's protocol. The qRT-PCR was conducted with the 7300SDS system from Applied Biosystems, using the SensiMixTM SYBR Kit according to the manufacturer's protocol. The concentration of the primers was optimized (400 nM final concentration), and a melting curve was performed to check the specificity of the primers. The primers used for the qRT-PCR are listed in Table S2 in the supplemental

material. To correct for small differences in the template concentration, *rpoD* was used as the housekeeping gene. The cycle in which the SYBR green fluorescence crossed a manually set cycle threshold (*CT*) was used to determine transcript levels. For each gene, the threshold was fixed based on the exponential segment of the PCR curve. The *CT* value of mutant was corrected for the housekeeping gene *rpoD* as follows: Δ*CT* = *CT* (mutant) - *CT* (*rpoD*); the same formula was used for the other genes investigated. The relative quantification (RQ) values were calculated by the following formula: RQ = $2^{-[\Delta CT(\text{mutant}) - \Delta CT(\text{wild type})]}$. If there was no difference in transcript levels between the mutant and the wild type, then RQ was equal to 1 (2^0) and logRQ was equal to 0. qRT-PCR analysis was performed in duplicate (technical replicates) on four independent RNA isolations (biological replicates). Statistically significant differences were determined for log-transformed RQ values by analysis of variance ($P < 0.05$), followed by Bonferroni post hoc multiple comparisons.

Siderophore detection assay

ChromoAzurolS (CAS) and M9 medium were prepared based on previously description (Schwyn & Neilands, 1987). M9 medium consists of 200mL 5X M9, 2mL 1M $MgSO_4$, 25mL 20% cassaminoacid, 0.1mL 1M $CaCl_2$, 15g Technical agar and water to 1L. The 5X M9 was made in advance, 500mL contained: 21.25g $Na_2HPO_4 \cdot 2H_2O$, 7.5g of KH_2PO_4, 1.25g of NaCl and 2.5g NH_4Cl. Orange halos around the colonies on the blue CAS agar plates are indicative of siderophore production.

Extracellular protease activity assay

Cells from different strains were washed with sterile MilliQ water and set to a final density of $1X10^9$ cells/mL. Then 5µL of this bacterial suspension was spotted on Skim Milk Agar plates (SMA, 1 Liter: 15g skim milk powder, 4g blood agar base, 0.5g yeast extract and 13.5g agar) and incubated at 25°C for 48 hrs. Extracellular protease activity was quantified by measuring the diameter of the transparent halo surrounding the bacteria colony.

Tensiometric analyses

Biosurfactant production was measured quantitatively by tensiometric analysis of the cell-free culture supernatant according to the method described before (de Bruijn & Raaijmakers, 2009b). Strains were inoculated in 6mL KB broth with the respective antibiotics and grown overnight at 25˚C. The overnight culture was washed with ample sterile milliQ and the cell density was set to $1.25x10^9$ cells/ml. 1.24 mL KB medium and 10µL of the bacteria were pipetted in each well of a 24-wells microtitre plate, resulting in a final concentration 10^7 cells/mL. Four replicates were made for each strain. The 24-well microtitre plates were incubated at 25˚C at 220rpm. When the cell density reached an OD_{600} of 0.6, the four replicate samples were pooled and the supernatants were collected. Biosurfactant production was measured quantitatively by tensiometric analysis of the supernatant at room temperature. For each culture and each condition,

measurements were done four times for each sample.

Nucleotide sequence accession number
The accession number for the genome sequence of *Pseudomonas fluorescens* SS101 is: NZ_AHPN00000000.

Acknowledgements

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Supplementary data

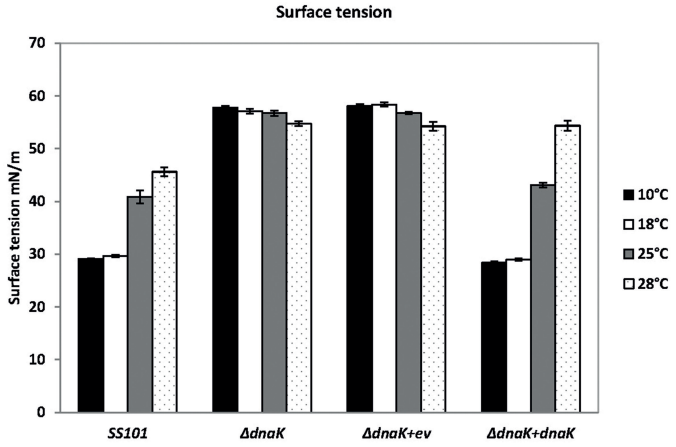

Figure S1. Effect of temperature on massetolide production in *Pseudomonas fluorescens* SS101. Surface tension of cell-free culture supernatant of wildtype strain SS101 was used as a proxy for production of the lipopeptide surfactant massetolide. Cell-free culture supernatants of the massetolide-deficient *dnaK* mutant, the *dnaK* mutant+EV (empty-vector control) and the *dnaK* mutant+pME6031-*dnaK* were used for comparison. Cells were grown at 10°C, 18°C, 25°C and 28°C respectively (220 rpm) in 24-well plates with 1.25 mL KB broth per well. Cell cultures at OD_{600}=0.6 were collected and spun down. Surface tensions of the cell supernatants were measured quantitatively by tensiometer at room temperature. Each sample was measured in four replicates and error bars represent the standard error of the mean.

Table S1. Bacterial strains used in this study.

Strain	Relative characteristics[1]	Reference
Pseudomonas fluorescens		
SS101	Wild type, Rif^r	(de Souza *et al.*, 2003)
Δ*phgdh*	Plasposon mutant, Km^r	This study
Δ*dnaK*	Plasposon mutant, Km^r	This study
Δ*prtR*	Plasposon mutant, Km^r	This study

[1] Rif^r: Rifampicin resistance; Km^r: Kanamycin resistance

Table S2. Primers used in this study.

Gene	Orientation	Primer sequence (5'-3')
massA	Forward	5'-GCTGTACAACATTGGCGGCT-3'
	Reverse	5'-GGTATGCAGTTGAGTGCGTAGC-3'
massB	Forward	5'-AACAACGACCGGAGATGCC-3'
	Reverse	5'-AAGGTGTGCAGCAAGTGATGG-3'
massC	Forward	5'-GTCGACCCTCAACGCGTCT-3'
	Reverse	5'-CCACCGACAGTTGGTCAAGC-3'
massAR	Forward	5'-GGCGCGCTTGAGGTAGGT-3'
	Reverse	5'-ACCGTGCCGCAAATTGC-3'
massBCR	Forward	5'-ATGCCGCCCGCTGAT-3'
	Reverse	5'-ACACCATCGAGAGCTACCTCAAG-3'

Chapter 4

Lipopeptide biosynthesis in *Pseudomonas fluorescens* is regulated by the protease complex ClpAP

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Abstract

Lipopeptides (LP) are structurally diverse compounds with potent surfactant and broad-spectrum antibiotic activities. In *Pseudomonas* and other bacterial genera, LP biosynthesis is governed by large multimodular nonribosomal peptide synthetases (NRPS). To date, relatively little is known about the regulatory genetic network of LP biosynthesis. This study provides evidence that the chaperone ClpA, together with the serine protease ClpP, regulates the biosynthesis of the LP massetolide in *Pseudomonas fluorescens* SS101. Whole-genome transcriptome analyses of *clpA* and *clpP* mutants showed their involvement in the transcription of the NRPS genes *massABC* and the transcriptional regulator *massAR.* In addition, transcription of genes associated with cell wall and membrane biogenesis, energy production and conversion, amino acid transport and metabolism, and pilus assembly were altered by mutations in *clpA* and *clpP*. Proteome analysis allowed the identification of additional cellular changes associated to *clpA* and *clpP* mutations. The expression of proteins of the citrate cycle and the heat shock proteins DnaK and DnaJ were particularly affected. Combined with previous findings, these results suggest that the ClpAP complex regulates massetolide biosynthesis via the pathway-specific, LuxR-type regulator MassAR, the heat shock proteins DnaK and DnaJ, and proteins of the TCA cycle. Combining transcriptome and proteome analyses provided new insights into the regulation of LP biosynthesis in *P. fluorescens* and led to the identification of specific missing links in the regulatory pathways.

Background

Lipopeptides (LPs) are biosurfactants produced by a variety of bacterial genera, including *Pseudomonas* and *Bacillus* (Raaijmakers *et al.*, 2006, Ongena & Jacques, 2008). LPs are composed of an (cyclic) oligopeptide moiety linked to a fatty acid tail (Raaijmakers et al., 2006). In beneficial *Pseudomonas* strains, LPs play a role in colonization of seeds (Nielsen *et al.*, 2005) and roots (Tran *et al.*, 2007), in defense against competing microorganisms and predatory protozoa (Mazzola *et al.*, 2009), and in swarming motility and biofilm formation (Raaijmakers *et al.*, 2010). LP biosynthesis is governed by large multi-modular nonribosomal peptide synthetases (NRPS) via a thiotemplate process (Finking & Marahiel, 2004, Raaijmakers et al., 2006). Compared to our understanding of LP biosynthesis, relatively little is known about the genetic networks involved in the perception of external signals and the signal transduction pathways that drive transcription of the LP biosynthesis genes. Here we focus on the regulation of LP biosynthesis in the plant growth-promoting rhizobacterium *Pseudomonas fluorescens* SS101. Strain SS101 produces the LP massetolide A, a 9-amino-acid cyclic peptide linked to 3-hydroxydecanoic acid (de Bruijn *et al.*, 2008, de Souza *et al.*, 2003). Massetolide A is produced in the early exponential growth phase and is essential for swarming motility and biofilm formation of strain SS101 (de Bruijn et al., 2008). Its biosynthesis is governed by three NRPS genes, designated *massA, massB*, and *massC* (de Bruijn et al., 2008).

To identify the genetic networks underlying regulation of massetolide biosynthesis, *P. fluorescens* strain SS101 was subjected to random mutagenesis. Screening of a library of approximately 7,500 random plasposon mutants resulted in the identification of four new regulatory genes, namely *phgdh*, *dnaK*, *prtR* and *clpA* (Song *et al.*, 2014a). In this recent study, we focused our functional analyses on *phgdh*, *dnaK* and *prtR*, but not on *clpA*. Independently from this work, *clpP* had been previously identified as a regulator of massetolide biosynthesis in *P. fluorescens* SS101 (de Bruijn & Raaijmakers, 2009). Hence, the aims of the present study were to i) study the role of ClpA in regulation of massetolide biosynthesis, and ii) analyse the ClpA regulon at the transcriptional and proteome level in order to narrow down the role of ClpP in regulating massetolide biosynthesis.

The ATP-dependent serine protease ClpP is highly conserved in eubacteria (Maurizi *et al.*, 1990) and has diverse functions, including intracellular proteolysis. ClpP associates with different ATPases that either recognize protein substrates directly or, alternatively, interact with substrates via so-called adaptor proteins (Kirstein *et al.*, 2009). Substrates are then unfolded and translocated to the proteolytic chamber of the ClpP protease (Gottesman, 2003). ClpP consists of two heptameric rings that form a barrel-shaped proteolytic core with the active sites hidden in an interior chamber (Reid *et al.*, 2001). The ATPases of ClpP that have been studied in detail in various bacterial genera include ClpX, ClpB, HslU and ClpA (Hoskins *et al.*, 1998, Gottesman, 1996). In strain SS101,

site-directed mutagenesis of *clpX* did not affect massetolide biosynthesis (de Bruijn & Raaijmakers, 2009), suggesting that ClpX does not act as the chaperone of ClpP in the regulation of massetolide biosynthesis. Therefore, the focus of our present study is on the role of the ClpAP complex in the regulation of massetolide biosynthesis. ClpA is formed as a hexameric chaperone ring complex and selects the target proteins for ClpP to degrade based on the N-end rule (Mogk *et al.*, 2007). Either misfolded or specifically tagged proteins are targeted by ClpA (Moore & Sauer, 2007). To unravel the cellular substrates of the ClpAP complex in *E.coli*, a proteomics approach (Flynn *et al.*, 2003) was adopted which revealed that several proteins involved in metabolism and energy production, cell motility and transport are potential cellular targets. In our study, we combined transcriptomic and proteomic analyses for both *clpA* and *clpP* mutants to identify putative substrates of the ClpAP complex with the ultimate goal to further elucidate the genetic regulation of massetolide biosynthesis in *P. fluorescens*.

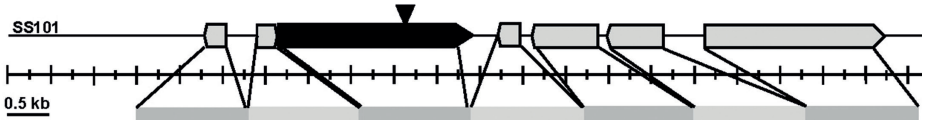
Results and discussion

Role of *clpA* in lipopeptide biosynthesis in *P. fluorescens* SS101

In *P. fluorescens* SS101, the *clpA* gene is 2271 bp with 89 to 98% identity to homologs in other *Pseudomonas* genomes (Figure 1). Based on the drop collapse assay, a mutation in the *clpA* gene abolishes massetolide production (Figure 2A). RP-HPLC analysis confirmed that the *clpA* mutant indeed did not produce detectable levels of massetolide A or its derivatives (Figure 2B). Complementation of the *clpA* mutant with the stable vector pME6031-*clpA* restored massetolide production to wild-type level, whereas the empty-vector control did not (Figure 2B). Massetolide biosynthesis is known to be essential for swarming motility of strain SS101 (de Bruijn et al., 2008). The *clpA* mutant was not able to swarm on soft agar (0.6% w/v; Figure 2C) and this phenotype was restored by complementation with pME6031-*clpA* (Figure 2C). In contrast to a mutation in *clpP*, no effects on growth were observed for the *clpA* mutant (Figure 2D). Collectively, these results indicated that *clpA* is required for massetolide biosynthesis in *P. fluorescens* SS101.

Transcriptome analysis

To further investigate the genetic basis for ClpAP-mediated regulation of massetolide biosynthesis, whole-genome transcriptome analyses were performed for the *clpA* (Figure S1A) and *clpP* (Figure S1B) mutants. Given the differences in growth kinetics between the mutants and wild-type SS101 (Figure 2D), cells were harvested in the exponential growth phase (OD_{600nm}=0.6). In the *clpA* mutant, transcription of 14 and 37 genes increased and decreased, respectively, by at least 2-fold (P_{FDR}<0.05) (Table S1). Apart from the massetolide biosynthesis genes, several of the differentially regulated genes were associated with energy production and conversion, amino acid transport and metabolism, cell wall and membrane biogenesis and pilus assembly. Several of the other differentially regulated genes could not be assigned to clusters of orthologous groups (COGs). Two pili gene clusters were significantly down-regulated in the *clpA* mutant. The first was the *csu* gene cluster (PflSS101_3282-3285) which is known to affect biofilm formation in *Acinetobacter baumannii* (de Breij *et al.*, 2009). The second was the type IVb pili gene cluster PflSS101_0648-0655 and the regulator *pprB* (Table S1). In *Pseudomonas aeruginosa*, type IVb pili are required for adhesion to abiotic surfaces and to eukaryotic cells (Bernard *et al.*, 2009). Further experiments will be needed to explore the functions of both pili gene clusters in *P. fluorescens* SS101.



Strain:	*cspD*	*clpS*	*clpA*	*infA*	*ate*	*aat*	*ftsK*
P. fluorescens SBW25	YP_002873361.1 85%	YP_002873360.1 100%	YP_002873359.1 98%	YP_002873358.1 100%	YP_002873356.1 90%	YP_002873357.1 94%	YP_002873355.1 98%
P. fluorescens Pf0-1	YP_349321.1 65%	YP_349320.1 100%	YP_349319.1 96%	YP_349318.1 100%	YP_349316.1 76%	YP_349317.1 91%	YP_349315.1 95%
P. protegens Pf-5	YP_260987.1 78%	YP_260986.1 97%	YP_260985.1 97%	YP_260984.1 100%	YP_260982.1 75%	YP_260983.1 92%	YP_260980.1 95%
P. syringae DC3000	NP_793138.1 87%	NP_793137.1 97%	NP_793136.1 94%	NP_793135.1 98%	NP_793133.1 74%	NP_793134.1 87%	NP_793132.1 90%
P. syringae B728a	YP_236255.1 70%	YP_236254.1 98%	YP_236253.1 94%	YP_236252.1 100%	YP_236250.1 76%	YP_236251.1 86%	YP_236249.1 90%
P. putida KT2440	NP_746140.1 74%	NP_746139.1 93%	NP_746138.1 93%	NP_746136.1 88%	NP_746135.1 70%	NP_746137.1 100%	NP_746134.1 78%
P. aeruginosa PAO1	NP_251312.1 79%	NP_251311.2 87%	NP_251310.1 89%	NP_251309.1 100%	NP_251307.1 77%	NP_251308.1 79%	NP_251305.1 79%

Figure 1. Genomic organization of *clpA* and flanking genes in *P. fluorescens* SS101. The *clpA* gene (PflSS101_ 3193) and flanking genes in *P. fluorescens* SS101 and the percentages of amino acid identity with their corresponding homologues in other *Pseudomonas* species and strains are indicated. The triangle indicates the position of the plasposon insertion in the *clpA* gene. Abbreviations: *cspD*: cold shock domain protein; *clpS*: ATP-dependent Clp protease adaptor protein; *clpA*: ATP-dependent Clp protease ATP-binding subunit; *infA*: translation initiation factor IF-1; *ate*: putative arginyl-tRNA-protein transferase; *aat*: leucyl/phenylalanyl-tRNA-protein transferase; *ftsK*: DNA translocase.

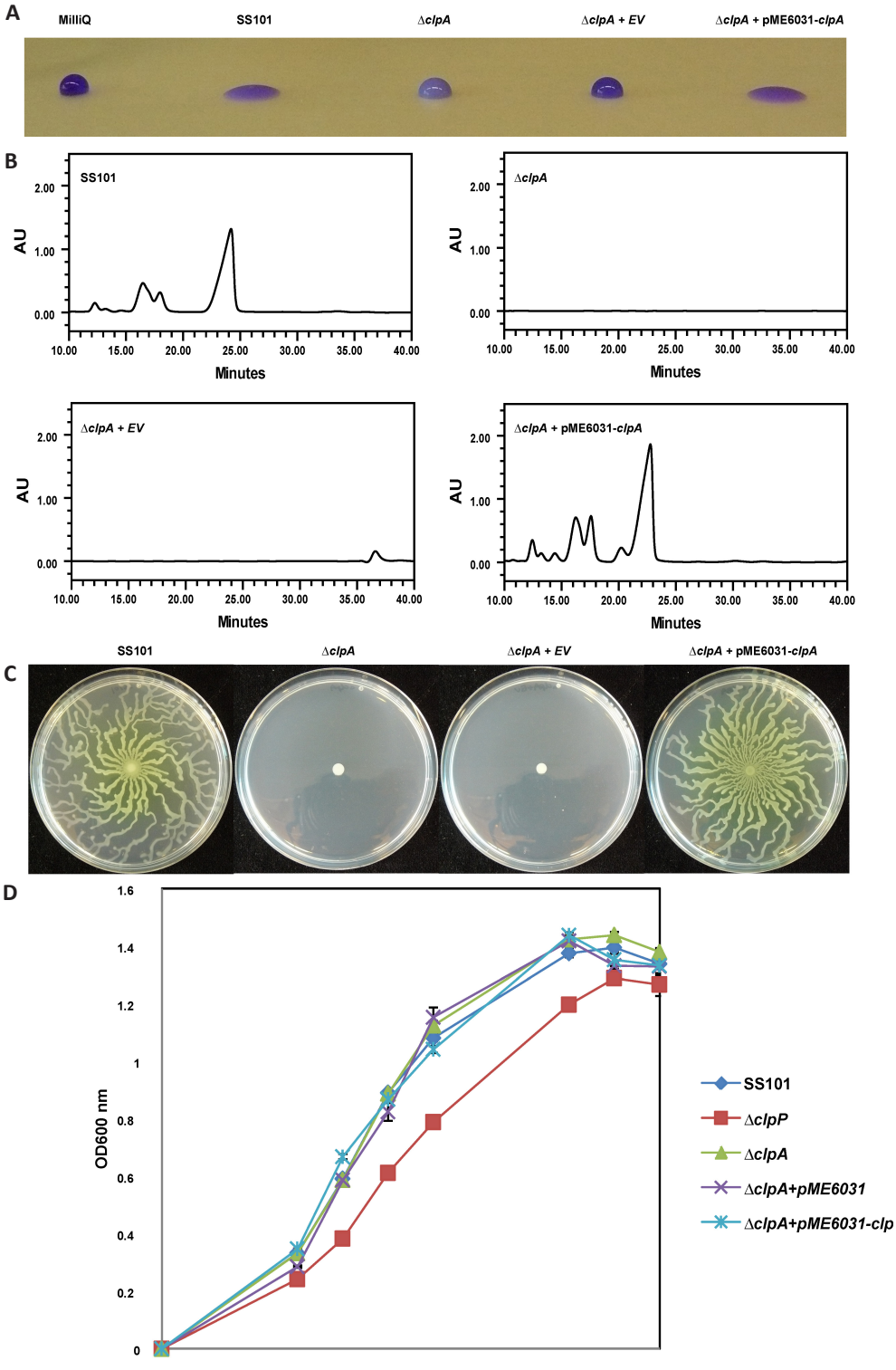

A
MilliQ
SS101
ΔclpA
ΔclpA + EV
ΔclpA + pME6031-clpA
B
SS101
ΔclpA
ΔclpA + EV
ΔclpA + pME6031-clpA
AU
Minutes
C
SS101
ΔclpA
ΔclpA + EV
ΔclpA + pME6031-clpA
D
OD600 nm
SS101
ΔclpP
ΔclpA
ΔclpA+pME6031
ΔclpA+pME6031-clp

Figure 2. Phenotypic and chemical analyses of *P. fluorescens* strain SS101, and its *clpA* mutant.
(**A**) Drop collapse assay with cell suspensions of wild-type strain SS101, *clpA* plasposon mutant, *clpA* mutant + pME6031 (empty vector control) and *clpA* mutant + pME6031-*clpA*. Bacterial cultures grown for 2 days at 25°C on KB agar plates were suspended in sterile water to a final density of $1x10^{10}$ cells/ml. 10-µl droplets were spotted on parafilm and crystal violet was added to the droplets to facilitate visual assessment. A flat droplet is a highly reliable proxy for the production of the surface-active lipopeptide massetolide A. (above)
(**B**) RP-HPLC chromatograms of cell-free culture extracts of the wild-type strain SS101, *clpA* plasposon mutant, *clpA*+pME6031 (empty vector control) and *clpA*+pME6031-*clpA* as described in panel A. The wild-type strain SS101 produces massetolide A (retention time of approximately 23-25 min) and various other derivatives of massetolide A (minor peaks with retention times ranging from 12 to 18 min) which differ from massetolide A in the amino acid composition of the peptide moiety. (above)
(**C**) Swarming motility of the wild-type strain SS101, *clpA* plasposon mutant, *clpA* mutant + pME6031 (empty vector control) and *clpA* mutant + pME6031-*clpA* on soft (0.6% wt/vol) agar plates. Five microliter ($1x10^{10}$ cells/ml) of washed cells from overnight cultures was spot-inoculated in the center of a soft agar plate and incubated for 48-72 h at 25ºC.
(**D**) Growth of the wild-type SS101 strain, *clpA* plasposon mutant, *clpA* mutant + pME6031 (empty vector control), *clpA* mutant + pME6031-*clpA* and *clpP* site-directed mutagenesis mutant in liquid medium at 25ºC. The optical density of the cell cultures was measured spectrophotometrically (600 nm) at different time points. Mean values of four biological replicates are given; the error bars represent the standard error of the mean.

With 195 and 154 genes significantly up and down regulated, respectively, the *clpP* mutation had a much bigger impact, as expected, on the overall gene expression in strain SS101 than a mutation in *clpA* (Table S2, Figure S1B). Combining the transcriptome data of the *clpA* and *clpP* mutants revealed that seven and thirteen genes were up and down-regulated, respectively, in both mutants (Figure 3). These include the massetolide biosynthesis genes *massA*, *massB, massC* and their flanking genes consisting of the LuxR-type transcriptional regulator *massAR* and the efflux-associated genes PflSS101_3398, PflSS101_2189 and PflSS101_2190. Among the genes differentially regulated in both *clpA* and *clpP* mutants were also the *thiF_moeB* gene cluster (PflSS101_3967-3970) as well as genes encoding a FAD-binding domain protein (PflSS101_0033) and an auto-inducer-binding LuxR-type transcriptional regulator (PflSS101_4691) (Figure 3). Expression of the previously identified regulatory genes of massetolide biosynthesis, *phgdh*, *dnaK*, and *prtR* (Song et al., 2014a), was not affected in the *clpA* and *clpP* mutants. This suggests that, at the transcriptional level, *clpAP*-mediated regulation of massetolide biosynthesis operates downstream or operates independently from these other regulatory genes.

Table 1. Regulator and chaperon proteins differentially expressed in the *clpP* mutant of *Pseudomonas fluorescens* SS101.

Locus	Gene	Gene description	Fold changes in Δ*clpP*/SS101
PflSS101_1716	*cysB*	HTH-type transcriptional regulator CysB	1.25 up
PflSS101_3936		transcriptional regulator, GntR family	1.35 up
PflSS101_4330	*mvaT*	transcriptional regulator MvaT	1.26 up
PflSS101_4600	*cbrB*	two-component response regulator CbrB	1.50 up
PflSS101_5275	*rnk*	regulator of nucleoside diphosphate kinase	1.65 up
PflSS101_1812	*htpG*	chaperone protein HtpG	1.2 up
PflSS101_4373	*groL*	chaperonin GroL	1.22 up
PflSS101_4374	*groS*	chaperonin GroS	1.32 up
PflSS101_4632	*dnaJ*	chaperone protein DnaJ	1.21 up
PflSS101_4633	*dnaK*	chaperone protein DnaK	1.32 up

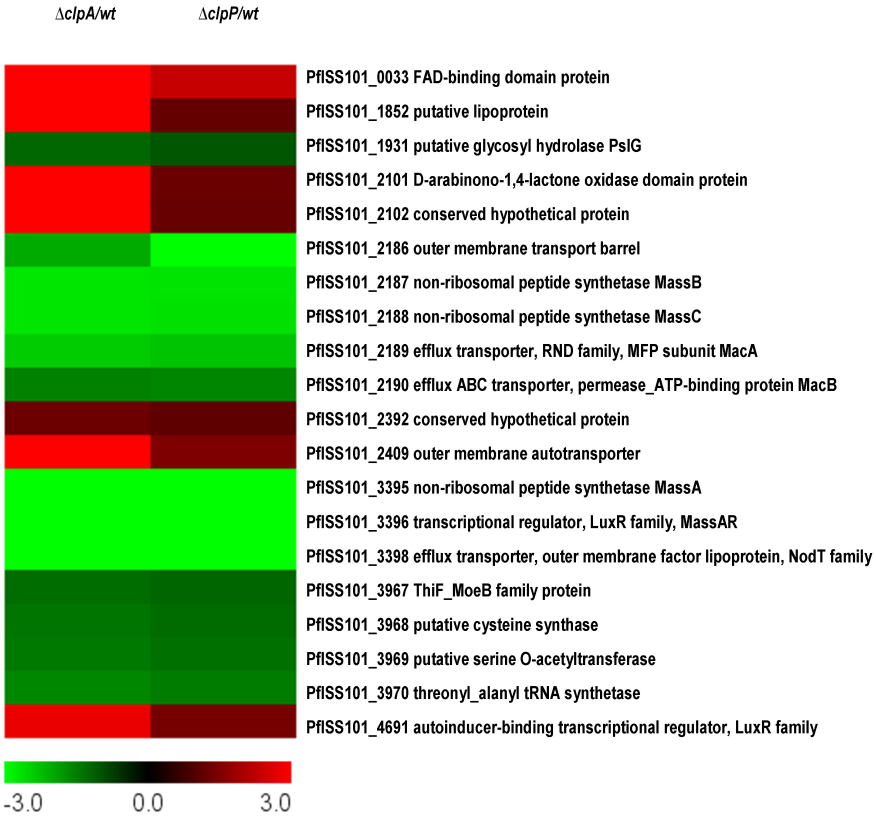


Figure 3. Heatmaps showing log_2-fold changes in the expression of genes that are differently expressed in the *clpA* or *clpP* mutants of *Pseudomonas fluorescens* SS101. See supplementary Tables S1 and S2 for the list of all genes differentially regulated in the *clpA* or *clpP* mutant versus wild-type SS101.

Proteome analysis

Total cell proteomic analyses were performed to further decipher the potential cellular substrates and target proteins of ClpAP (Figure S2). The culture conditions and 'harvest' time of the bacterial cells (OD_{600}=0.6) were identical to those used in the transcriptome analyses described above. It should be noted that the ClpAP system is a degradative protease thereby complicating the interpretation of proteomics data. While transcriptomics can validly argue that mRNAs (and hence proteins) are up- or down-regulated, the higher abundance of a particular protein in the *clpA* and or *clpP* mutants can also be due to an inherent up- or down-regulation by other modulated pathways. Hence, the proteomics results described below should be interpreted with caution.

Proteins differentially expressed in the clpA mutant or clpP mutant

iTRAQ-based proteome analyses allowed the identification of a total of 596 proteins in the *clpA* mutant (Table S3): 68 proteins were significantly up-regulated (Fold change > 1.2) while 132 were down-regulated (Table S3). Gap2 (PflSS101_4355), encoding a

glyceraldehyde-3-phosphate dehydrogenase, was up-regulated in the *clpA* mutant, which was consistent with the earlier report (Flynn et al., 2003) that reported a similar GapA protein as one of the substrates of ClpAP in *E. coli*. All three protein groups from the 'intracellular trafficking and secretion' COG category were up-regulated in the *clpA* mutant, including SecA, SecB, and the Tol-Pal system protein TolB (Figure S2A, Table S3).

In line with the findings in *E. coli* (Flynn et al., 2003), we observed that the cell division protein FtsZ and the isocitrate lyase AceA were up-regulated in the *clpP* mutant (Figure S2B; Table S4), suggesting that these proteins might be substrates of ClpP in strain SS101. Moreover, we detected five transcriptional regulators and five chaperons that were uniquely up-regulated in the *clpP* mutant (Table 1). One of the up-regulated transcriptional regulators was MvaT (PflSS101_4330), which is known to regulate the biosynthesis of specific secondary metabolites in the rhizobacterium *Pseudomonas protegens* CHA0 (Baehler *et al.*, 2006). Furthermore, the heat shock proteins DnaK and DnaJ, the chaperonin GroS, GroL and the chaperon HtpG were significantly up-regulated in the *clpP* mutant. Also CheA, a histidine kinase that mediates chemotaxis signaling events in many prokaryotes (Stewart, 2010), was 1.49-fold up-regulated, suggesting it may be a substrate of ClpP in strain SS101.

Proteins differentially expressed in both clpA and clpP mutants

In both *clpA* and *clpP* mutants, 32 and 39 proteins were up- and down-regulated, respectively (Table 2, Table S5). The most up-regulated was CspD (PflSS101_3195), a gene encoding one of the cold shock protein CspA family members in *E. coli.* CspD is known to be induced by nutritional deprivation (Yamanaka & Inouye, 1997). Moreover, the response regulator CbrB and the transcriptional regulator GntR were up-regulated in both mutants. The CbrA-CbrB two-component system is known to control the utilization of different carbon and nitrogen sources in *P. aeruginosa* (Nishijyo *et al.*, 2001) and affects chemotaxis, stress tolerance and biofilm development in *Pseudomonas putida* (Amador *et al.*, 2010). GntR is a transcriptional regulator that controls antibiotic production in both *Streptomyces* and *Serratia* (Hillerich & Westpheling, 2006, Fineran *et al.*, 2005). None of these proteins and their corresponding genes were found in genome-wide screening for massetolide-deficient mutants, except DnaK (Song et al., 2014a). In our proteome analyses, the DnaK protein was found at higher concentrations in the *clpP* mutant and its chaperon DnaJ protein was up-regulated in both *clpA* and *clpP* mutants. Given that DnaK and DnaJ also regulate putisolvin biosynthesis in *P. putida* (Dubern *et al.*, 2005), our results suggest that ClpAP regulates LP biosynthesis in multiple *Pseudomonas* species at least in part, via DnaK and DnaJ (Figure 4).

Table 2. Up-regulated proteins in both *clpA* and *clpP* mutants of *Pseudomonas fluorescens* SS101.

Locustag	Gene	Gene descriptions	Δ*clpA*/SS101	Δ*clpP*/SS101
PflSS101_0002	*dnaN*	DNA polymerase III, beta subunit	1.3	1.3
PflSS101_0021	*qor*	NADPH_quinone reductase	1.25	1.6
PflSS101_0364	*secB*	protein-export chaperone SecB	1.34	1.42
PflSS101_0509	*thiC*	thiamine biosynthesis protein ThiC	1.33	1.28
PflSS101_0546	*rnr*	ribonuclease R	1.27	1.27
PflSS101_0920	*hisC_1*	histidinol-phosphate transaminase	1.3	1.2
PflSS101_0926	*mqo_1*	malate_quinone-oxidoreductase	1.32	1.21
PflSS101_1161	*argG*	argininosuccinate synthase	1.3	1.2
PflSS101_1203		TIGR00730 family protein	1.22	1.22
PflSS101_1209	*fpr_2*	ferredoxin--NADP+ reductase	1.28	1.24
PflSS101_1348	*fabD*	acyl-carrier-protein S-malonyltransferase	1.26	1.32
PflSS101_1554		LamB_YcsF family protein	1.25	1.27
PflSS101_1626		short-chain alcohol dehydrogenase family protein	1.53	1.23
PflSS101_1652	*cmk*	cytidylate kinase	1.35	1.36
PflSS101_1729		3-deoxy-7-phosphoheptulonate synthase	1.28	2
PflSS101_2196		AP endonuclease, family 2	1.65	2.12
PflSS101_3195		cold shock domain protein CspD	2.14	3.15
PflSS101_3348	*bkdA2*	2-oxoisovalerate dehydrogenase E1 component, beta subunit	1.26	1.23
PflSS101_3776		flagellin domain protein	1.21	2.14
PflSS101_3786	*phhA*	phenylalanine-4-hydroxylase	1.24	1.81
PflSS101_3936		transcriptional regulator, GntR family	1.25	1.35
PflSS101_4181		conserved hypothetical protein	1.2	1.2
PflSS101_4298	*tolB*	Tol-Pal system beta propeller repeat protein TolB	1.33	1.29
PflSS101_4316		PF04461 family protein	1.21	1.55
PflSS101_4394	*thrC*	threonine synthase	1.29	1.43
PflSS101_4600	*cbrB*	two-component response regulator CbrB	1.25	1.5
PflSS101_4631	*dapB*	dihydrodipicolinate reductase	1.5	1.55
PflSS101_4632	*dnaJ*	chaperone protein DnaJ	1.26	1.22
PflSS101_4676		conserved hypothetical protein	1.31	1.21
PflSS101_4945	*rpsU*	ribosomal protein S21	1.25	1.25
PflSS101_5275	*rnk*	regulator of nucleoside diphosphate kinase	1.52	1.65
PflSS101_5280	*lysA*	diaminopimelate decarboxylase	1.23	1.27

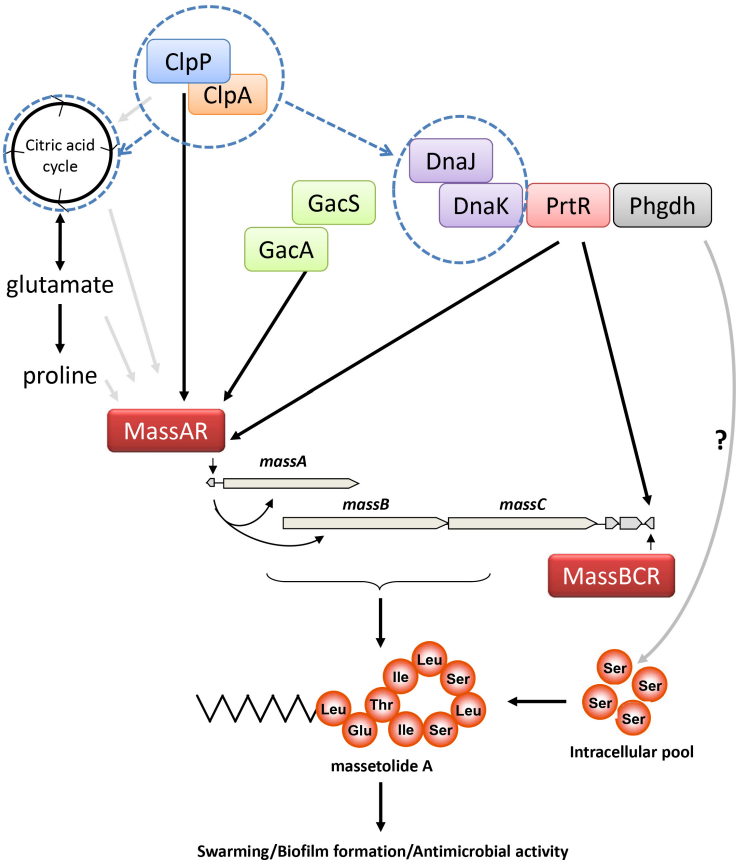


Figure 4. Proposed model for the genetic regulation of massetolide biosynthesis in *P. fluorescens* strain SS101. The darkly shaded arrows are based on experimental data obtained earlier (Song et al., 2014b) and in this study; the lightly shaded arrows are hypothetical and not based on experimental data. The blue dashed arrows and circles represent translational regulation whereas the other arrows represent transcriptional regulation.

TCA cycle proteins were expressed differently in both clpP and clpA mutants

Our proteome analyses also revealed that several proteins from the TCA cycle were differentially expressed in both the *clpA* and the *clpP* mutants (Figure S3). Five proteins were down-regulated and two were up-regulated in the *clpA* mutant. Similar numbers of down-regulated (6) and up-regulated (2) proteins were found in the *clpP* mutant (Figure S3). In the TCA cycle, PckA (PflSS101_0285) encodes phosphoenolpyruvate carboxykinase ATP and transfers oxaloacetate to phosphoenolpyruvate. This protein was 1.20 up- and 1.47 down-regulated in the *clpA* and *clpP* mutants, respectively. Mqo_1 (PflSS101_0926), a malate quinone oxidoreductase, was up-regulated in both mutants. Malate quinone oxidoreductase is known to be essential for growth on ethanol or acetate in *Pseudomonas aeruginosa* (Kretzschmar *et al.*, 2002). It is also required for virulence of *Pseudomonas syringae* pv. tomato strain DC3000 on *Arabidopsis thaliana* (Mellgren *et al.*, 2009). Its function in *P. fluorescens* SS101, however, is not yet known.

Conclusions

ClpA is a chaperon protein that is highly conserved in bacteria and eukaryotes (Wong & Houry, 2004, Yu & Houry, 2007). Together with the serine protease ClpP, it plays an important role in intracellular refolding and degradation of proteins, an essential process for the viability and growth of cells. In this study, we cloned and sequenced *clpA* from the plant growth-promoting bacterium *P. fluorescens* strain SS101 and showed that *clpA* plays an important role in the regulation of massetolide biosynthesis. The combined results of the transcriptomic and proteomic analyses suggest that the ClpAP complex regulates massetolide biosynthesis via the pathway-specific LuxR-type transcriptional regulator MassAR, the heat shock proteins DnaK and DnaJ and via proteins involved in the TCA cycle. These findings extend our previous regulatory model for LP biosynthesis in *P. fluorescens* SS101 (Figure 4) which, to a large extent, may also apply to the regulatory networks of LP biosynthesis in other *Pseudomonas* species and strains.

Methods

Bacterial strains and culture conditions

P. fluorescens strain SS101 and its *clpP* and *clpA* mutants were cultured in King's medium B (KB) broth at 25°C. The *clpA* and *clpP* mutants were obtained in our previous studies (de Bruijn & Raaijmakers, 2009, Song et al., 2014a). *Escherichia coli* strain DH5α was the host for the plasmids used for genetic complementation. *E. coli* strains were grown on Luria-Bertani (LB) plates or in LB broth amended with the appropriate antibiotics.

Identification of the clpA cluster

clpA was identified by partial sequencing of the regions flanking the plasposon insertion as described by Song et al (Song *et al.*, 2014b). The complete flanking regions of *clpA* were obtained from the genome sequence of *P. fluorescens* SS101 (Loper *et al.*, 2012). Open reading frames (ORFs) were identified with the Softberry FGENESB program (http://www.softberry.com/berry.phtml). The ORFs were analyzed using BlastX in the NCBI database and Pseudomonas.com (http://pseudomonas.com). For genetic complementation, the pME6031-*clpA* construct was generated according to methods described previously (de Bruijn & Raaijmakers, 2009). Briefly, a 2,870-bp fragment, including the promoter and terminator, was subcloned into the shuttle vector pME6031 and transformed into. *E. coli* DH5α. The pME6031-*clpA* construct was subsequently electroporated into the *clpA* plasposon mutant of *P. fluorescens* SS101. Transformed cells were plated on KB supplemented with tetracycline (25 µg/ml), and the presence of pME6031-*clpA* was verified by PCR analysis with primers specific for pME6031.

Lipopeptide extraction and RP-HPLC separation

Massetolide extractions and RP-HPLC analysis were performed as described earlier (de Bruijn et al., 2008, de Bruijn & Raaijmakers, 2009, Song et al., 2014a). Briefly, *Pseudomonas* strains were grown on *Pseudomonas* isolation agar plates (Pseudomonas agar 38g/L, glycerol 10g/L) for 48 h at 25°C. The cells were suspended in sterile demineralized water (~40 ml per plate), transferred to 50 mL tubes, shaken vigorously for 2 min and then centrifuged (30 min, 5292 g, 4°C). The culture supernatant was transferred to a new tube and acidified to pH 2.0 with 9% HCl. The precipitate was recovered by centrifugation (30 min, 5292 g, 4°C) and washed three times with acidified dH_2O (pH 2.0). It was then resuspended in 5 mL dH_2O and the pH adjusted to 8.0 with 0.2 M NaOH until complete dissolution. The solution was centrifuged (30 min, 5292 g, 4°C) and the supernatant transferred to a new tube, subjected to lyophilisation and RP-HPLC analysis according to methods described previously (Song *et al.*).

Swarming motility

Swarming motility assays of the wild-type and mutants strains were performed as described earlier (Song et al., 2014b). Swarming motility of the wild-type SS101 strain and the mutants was assessed on soft [0.6% wt/vol] standard succinate agar medium (SSM) consisting of 32.8 mM K_2HPO_4, 22 mM KH_2PO_4, 7.6 mM $(NH_4)_2SO_4$, 0.8 mM $MgSO_4$, and 34 mM succinic acid. The pH of the medium was adjusted to 7 with NaOH. Cells from overnight cultures of the wild-type and mutant strains were washed three times with 0.9% NaCl, and 5 µL of the washed cell suspensions ($1X10^{10}$ cells/ml) was spot inoculated in the centre of the soft SSM agar plate and incubated for 48-72 h at 25°C.

Transcriptome analysis

The wild-type SS101 strain and the *clpA* and *clpP* mutants were grown in KB broth in 24-well plates, and harvested for RNA isolation at an OD_{600nm}=0.6. For each strain, three biological replicates were used. Total RNA was extracted with Trizol reagent (Invitrogen) and further purified with the NucleoSpin RNA kit. A tiling microarray for *P. fluorescens* SS101 was developed by the Dutch Genomics Service & Support Provider, University of Amsterdam (UvA, Amsterdam, the Netherlands). In total, 134,276 probes (60-mer) were designed with, in general, a gap of 32 nucleotides between adjacent probes on the same strand and an overlap of 14 nucleotides for both strands. In addition, 5,000 custom negative control probes were hybridized and used as an internal control to validate the designed probes in a CGH experiment of 4 arrays. Probes were annotated and assembled into probe sets for known genes based on location information retrieved from the Pathosystems Resource Integration Center (PATRIC, http://patricbrc.org). Probes outside of known gene sequences were labeled as InterGenic Region (IGR). cDNA labelling was conducted as described previously (de Knegt *et al.*, 2013). Briefly, cDNA was synthesized in presence of Cy3-dUTP (Cy3) for the test samples and with Cy5-dUTP (Cy5) for the common reference. The common reference consisted of an equimolar

pool of the test samples (3 µg per sample). 5 µg of total RNA per reaction was used and yielded 1.5-2.5 µg cDNA for each sample with larger than 16 pmol of Cy3 or Cy5 dye per microgram. Hybridizations were performed as described elsewhere (Pennings *et al.*, 2011). Slides were washed according to the procedures described in the Nimblegen Arrays User's Guide - Gene Expression Arrays Version 5.0 and scanned in an ozone-free room with an Agilent DNA microarray scanner G2565CA (Agilent Technologies). Feature extraction was performed with NimbleScan v2.5 (Roche Nimblegen). Data pre-processing consisted of $\log_2$-transformation of the raw probe-intensity data, followed by a within slide Lowess normalization. Thus normalized sample (Cy3) channel intensities were summarized into probe sets values and normalized between arrays using the RMA (Robust Multi-Array Analysis) algorithm (Irizarry *et al.*, 2003). Analysis of the gene expression data was conducted using the Arraystar software. All results described were found to be significant using a false discovery rate of less than 5%.

Proteome analysis

The wild-type SS101 strain and the *clpA* and *clpP* mutants were grown in KB broth in 24-well plates, and cells were harvested for protein extraction at an OD_{600nm}=0.6. Three biological replicates were used for each strain. The cells were harvested by centrifugation and resuspended in 15 mL ice-cold 1 x PSB buffer containing the protease Inhibitor Cocktail from Sigma-Aldrich, as instructed by the manufacturer. The following steps were performed at 4 °C. The cells were disrupted twice in a French pressure cell press (SLM Instruments Inc) at 14,000 psi and centrifuged for 30 min at 47,000g. Protein concentration was determined using the Bradford assay followed by iTRAQ labeling in a 4-plex experiment according to the manufacturer's protocol (AB Sciex Pte. Ltd). Briefly, 100 µg of protein in 100-400 µL were successively reduced in the presence of 1 µL TCEP (tris(2-carboxyethyl)phosphine), alkylated using 2 µL 85 mM iodoacetamide, and hydrolyzed with 2.5 µg trypsin. A further addition of 2.5 µg trypsin 1 h after the initial addition of the protease was performed prior to an overnight incubation. Each of the reaction mixtures was then freeze-dried, redissolved in 100 µL 125 mM TEAB (triethylammonium bicarbonate) in 75% ethanol and transferred to one vial of iTRAQ reagent (4-plex, 114-117). After 1 h incubation, 100µL of H_20 was added followed by 15 min incubation in order to hydrolyze the excess of iTRAQ reagent. The resulting samples were pooled together and desalted using SepPak C18 cartridges (Waters Corporation). The pooled samples (800 µL) were diluted to 3.6 mL in 0.1% formic acid (FA) and loaded onto pre-wetted (95% acetonitrile (ACN) containing 0.1% FA) and equilibrated (0.1% FA) cartridges. After washing the loaded cartridges 5 times with 1 mL 0.1% FA, elution was performed in 1 mL 50% ACN/0.1% FA followed by 95% ACN/0.1% FA. Eluates were combined and evaporated to dryness.

The evaporated iTRAQ-labeled samples were resolubilized (10 µL) in the sample loading buffer (5 mM ammonium acetate containing 5% ACN) and injected (4.9 µL) using the partial loop mode on a liquid chromatograph (nanoAcquity UPLC system, Waters

Corporation) plumbed for two-pump trapping and two-dimensional strong-cation exchange and reversed-phase (SCX-RP) separation. Salt plugs (10, 20, 30, 40, 50, 80, 150, 200 mM ammonium acetate in 5% ACN, followed by 200 mM in 30% ACN and 350 mM in 50% ACN) were injected using the full loop mode. Sample and salt plugs were loaded in trap mode (SCXtrap-C18trap-waste) onto the SCXtrap column (18x20mm, 5 µm particle size, P/N 186003507) using the sample and loading buffer for 10 min at 5 µL/min. Subsequently, an analytical separation was performed in analytical mode (C18trap-C18Analytical-ESI source) at 400 nL/min with the following consecutive steps and gradient: 1% B (100% ACN, 0.1% FA) (0–1 min); 1–40% B (1-50 min); 40-60% B (50-65 min); 60-85% B (65-66 min); 85% B (66-70 min); 85-1% B (70-71 min).

The gradient flow from the nanoAcquity was delivered into the Nano ESI ion source of a Xevo Q-TOF mass spectrometer from Waters Corporation (source voltage 4 kV; source temperature 80ºC; cone voltage 35V; cone gas flow 20 L/h; nano flow gas 0.8 bar). Data were acquired in data dependent mode with one full scan (350-1400 m/z) followed by maximum 5 MS/MS scans (50-1800 m/z) on doubly and triply charged peptides only. External TOF mass calibration was performed prior to the UPLC-MS analysis. This was obtained by direct infusion of a solution containing 2 g/L sodium iodide in 50% isopropanol, and data acquisition in TOF-MS mode over the *m/z* range 50–2000.

Proteome data analysis

Raw data files were treated using the trans-proteomic pipeline (TPP) software package for proteomic data analysis supplied by the Seattle Proteome Centre (Keller *et al.*, 2005). The processing of data through the TPP modules was automated by in-house java-based software. Initially, .raw files were converted into uncentroided mzXML files using MSConvert. Before search all data was centroided and processed to only keep the top 100 peaks in each fragment spectra. Centroided data was then analysed using X!tandem with native scoring. Search hits from each individual replicate were assigned probabilities using Peptide Prophet (Kessner *et al.*, 2008) utilizing the semi-parametric model, at this stage each technical-replicate was assigned a unique experiment ID to allow iProphet (Craig & Beavis, 2004) to utilize the number of replicate experiments model. Libra (TPP module) was then used to extract iTRAQ reporter ion signals from the uncentroided data, in each replicate the four different iTRAQ reporter channels were normalized to account for 25% of the total signal.

Each set of technical replicates were then combined into a single output pep.xml using iProphet (Keller *et al.*, 2002)2002 and final protein lists were assembled using Protein Prophet (Shteynberg *et al.*, 2011) and Libra was used to calculate iTRAQ protein ratios. Parameters used for analysis were as follows; X!tandem searches were ran against the P. fluorescens SS101 amino acid sequence database, concatenated to its own reversed sequences for use as decoy hits. Searches used trypsin specificity, a precursor ion tolerance of 50 ppm, a fragment monoisotopic tolerance of 0.4 Da and the following

post-translational modifications were assigned; fixed carbamidomethyl cystein, fixed iTRAQ (N-term), fixed iTRAQ (K), variable oxidation (M), variable iTRAQ (Y), variable phosphorylation (S/T). Libra protein ratios were extracted using intensity weighted average, using normalization by sum of reagent profiles, minimum reporter ion intensity of 20 and a reporter ion mass tolerance of 0.05.

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Supplementary data

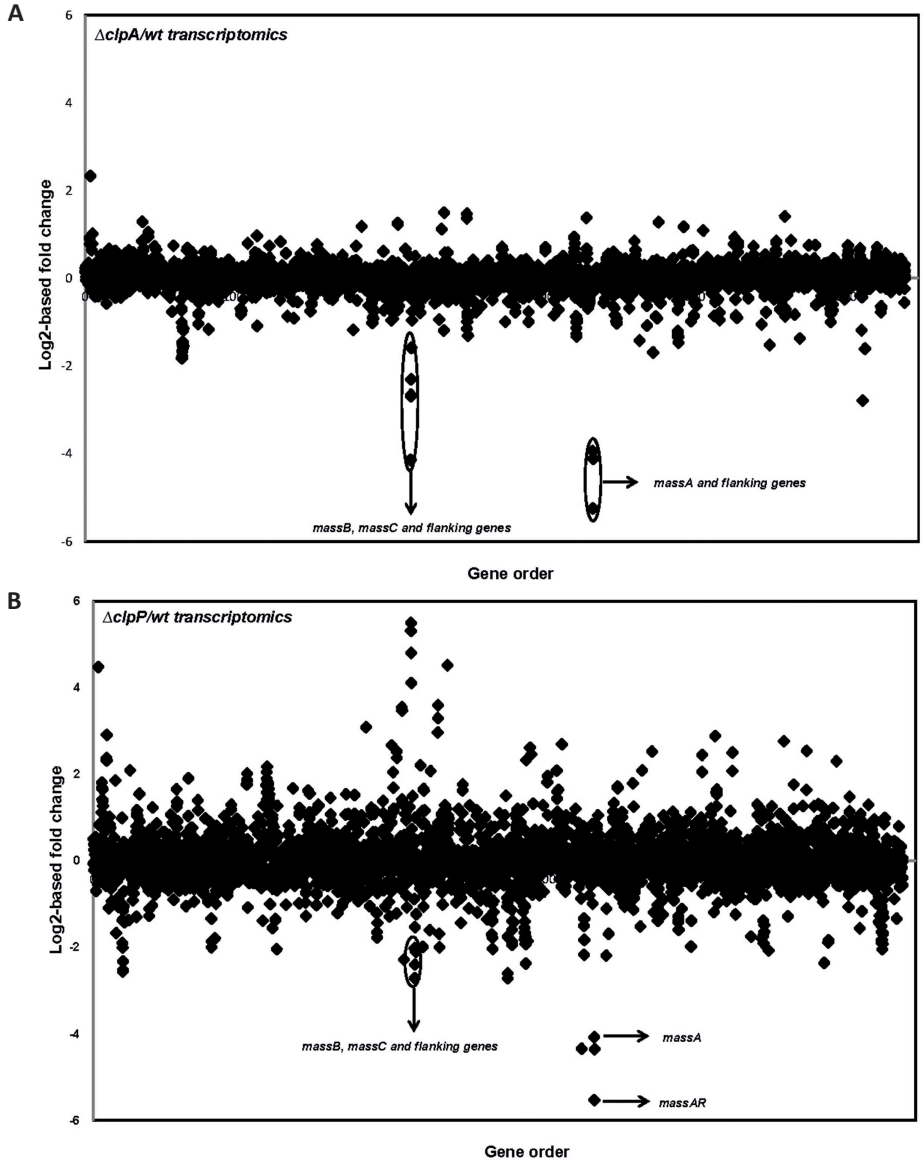

Figure S1. Differential gene transcription between the wild-type *P. fluorescens* SS101 strain and the *clpA* (A) or *clpP* (B) mutant at exponential phase (OD600 = 0.6), assessed by microarray analyses. The transcription chart shows log2-based fold changes of transcripts of *clpA* or *clpP* mutant compared to the wild-type strain SS101. Each dot in the chart represents each of the 5374 annotated genes in the SS101 genome with the x-axis showing gene order, and the y-axis showing the log2 of relative transcripts abundance for each gene in the *clpA* or *clpP* mutant compared to the wild-type strain SS101. Gene clusters whose members are discussed in the main text are shown.

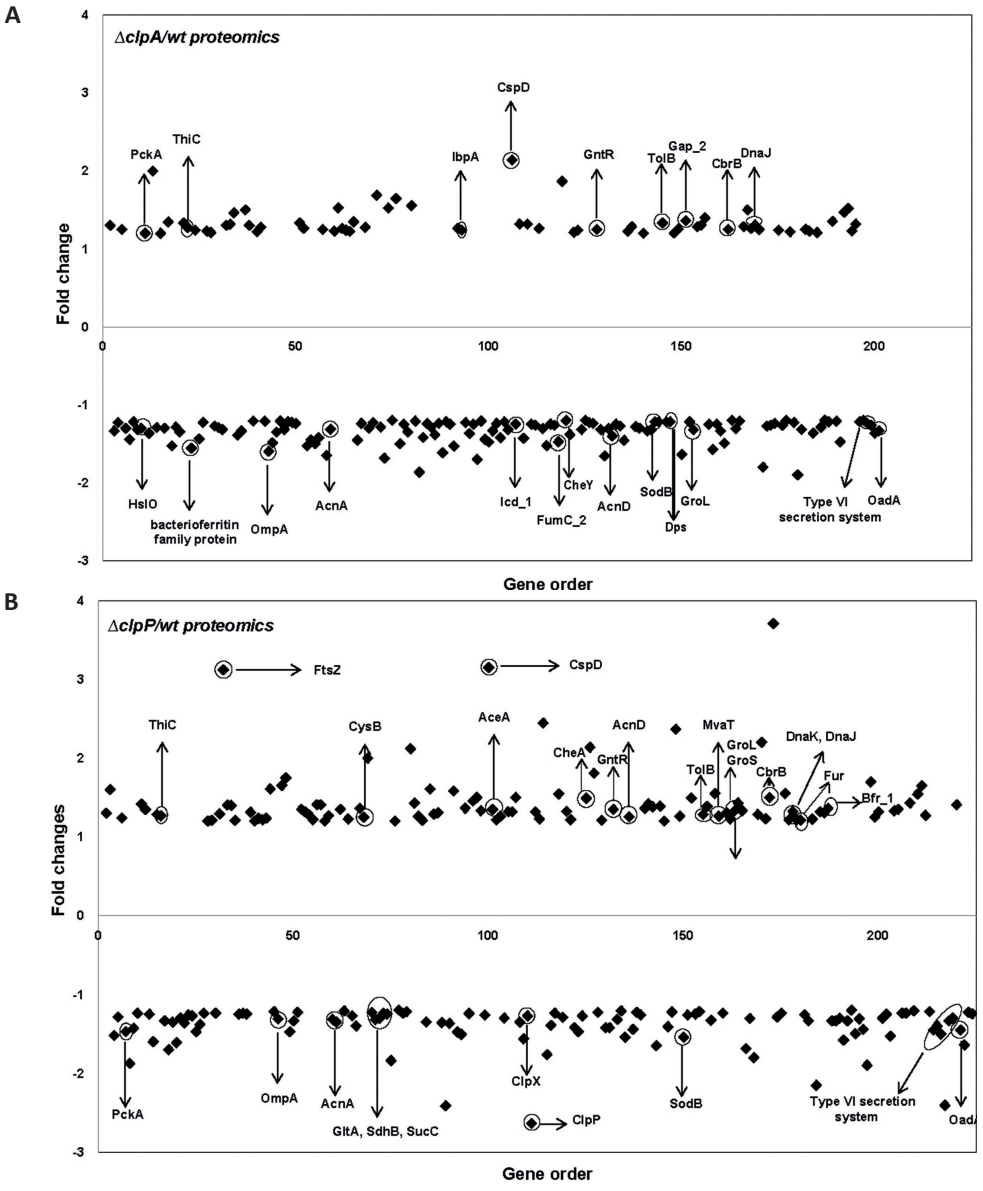

Figure S2. Differential protein expression between wild-type *P. fluorescens* SS101 and the *clpA* (A) or the *clpP* (B) mutant at exponential phase (OD600 = 0.6), assessed using isobaric tag labeling for relative and absolute quantitation (iTRAQ) experiments. The expression chart shows fold changes of protein expression in the *clpA* or *clpP* mutant compared to the wild-type strain SS101. Each dot in the chart represents the 200 and 223 proteins that significantly accumulated in the *clpA* and *clpP* mutants, respectively. The x-axis shows gene order and the y-axis shows fold changes.

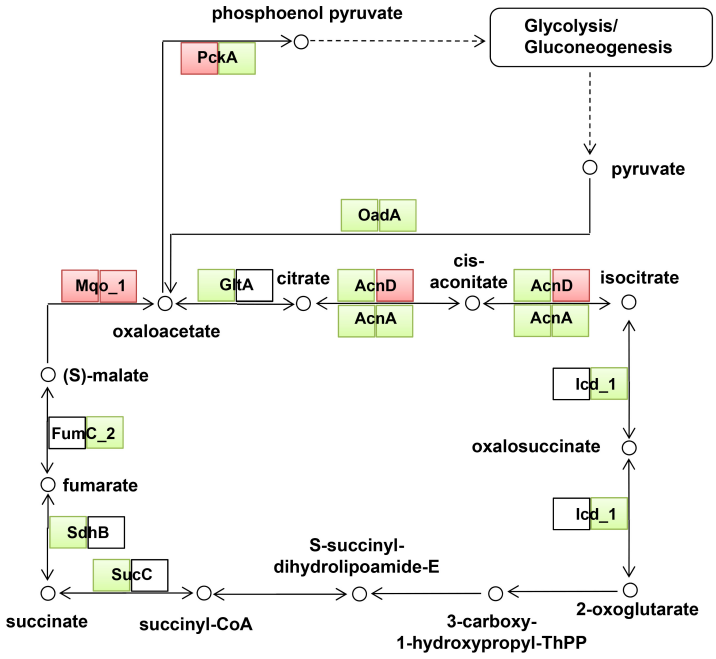

Figure S3. TCA cycle pathway of *P. fluorescens* SS101 (adjusted from KEGG with *P. fluorescens* A506, the most related strain of SS101). Red boxes indicate up-regulation; green boxes indicate down-regulation; empty boxes stand for "not detected". The left and right boxes stand for protein expression in the *clpA* and *clpP* mutants, respectively.

Supplementary tables are available on the website:
http://www.biomedcentral.com/1471-2180/15/29/additional

Chapter 5

Transcriptional and metabolic responses at the interface of *Pseudomonas*-protozoa interactions

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Abstract

Soil-dwelling bacteria of the genus *Pseudomonas* produce lipopeptide surfactants (LPs) with broad-spectrum antimicrobial activities. Recent studies suggested that LPs provide protection to *P. fluorescens* against grazing by the predatory protozoa *Naegleria americana*, both in vitro and in rhizosphere environments (Mazzola *et al.*, 2009). These findings documented a new natural function of LPs and suggested that *Pseudomonas*-protozoa interactions activate an antipredator response in prey populations. Here, genome-wide transcriptome analysis revealed that upon protozoan grazing, 55 genes were up-regulated and 73 genes were down-regulated in *P. fluorescens* strain SS101. Among the up-regulated genes were the LP biosynthesis genes *massABC,* genes involved in alkane degradation and in putrescine catalysis. Subsequent assays revealed that putrescine induced trophozoite encystment and adversely affected cyst viability of *N. americana*. MALDI imaging mass spectrometry (IMS) and live colony NanoDESI mass spectrometry further showed, real time, site-specific LP production at the interface of *Pseudomonas*-protozoa interactions. Identical transcriptional and metabolic responses were observed in the interaction of *P. fluorescens* strain SBW25 with *N. americana*, including the induction of LP and putrescine biosyntheses. Collectively, this multifaceted study provides new insights in common and also strain-specific transcriptional and metabolic responses in bacteria-protozoa interactions, including those responses that may contribute to microbial survival in the highly competitive rhizosphere environment.

Introduction

The rhizosphere is home to diverse organisms including bacteria, fungi, oomycetes, nematodes, protozoa, algae, viruses, archaea and arthropods (Bonkowski *et al.*, 2009, Buee *et al.*, 2009, Mendes *et al.*, 2013, Philippot *et al.*, 2013). Elevated densities of microorganisms in the rhizosphere leads to concomitant increases in the populations and feeding activities of their predators (Taylor, 1978). Predation plays a significant role in shaping the structure of bacterial communities (Ronn *et al.*, 2002, Bonkowski & Brandt, 2002). In turn, bacteria possess various defense strategies to resist or evade predation by protozoa via both intracellular and extracellular adaptations (Matz & Kjelleberg, 2005). Intracellular adaptations include survival and replication of bacteria inside the protozoan cell (Brown & Barker, 1999). Extracellular avoidance mechanisms include altered cell morphology (Hahn & Hofle, 1998, Hahn *et al.*, 1999, Pernthaler *et al.*, 2004), increased bacterial motility (Matz & Jurgens, 2005), biofilm formation (Matz *et al.*, 2004) and production of bioactive compounds (Matz *et al.*, 2004, Cosson *et al.*, 2002, Pukatzki *et al.*, 2002).

For *Pseudomonas* species, hydrogen cyanide (HCN), 2,4-diacetylphloroglucinol (2,4-DAPG) and pyrrolnitrin (PRN) were shown to contribute to defense against protozoa (Jousset *et al.*, 2010, Gallagher & Manoil, 2001). Also extracellular proteases inhibit protozoan predation in *Pseudomonas* (Jousset *et al.*, 2006) as well as in *Vibrio cholerae* (Vaitkevicius *et al.*, 2006, Niu *et al.*, 2010). We previously assessed the function of lipopeptide surfactants (LPs) as a bacterial defense mechanism against protozoan predation: LPs were shown to limit protozoan grazing of *Pseudomonas fluorescens* both *in vitro* and *in situ* (Mazzola *et al.*, 2009). Interestingly, protozoa-*Pseudomonas* interactions led to enhanced transcription of LP biosynthesis genes (Mazzola *et al.*, 2009). These results suggested that bacteria can modulate the production of secondary metabolites in response to protozoan predators. However, evidence that LPs are actually produced during protozoa-*Pseudomonas* interactions is lacking. Also knowledge of the overall chemistry and transcriptional responses at the bacteria-protozoa interaction site remains elusive. The aim of this study was to unravel predation-mediated responses at the interface of *Pseudomonas*-protozoa interactions. To that end, we conducted whole-genome transcriptome, MALDI-TOF-based imaging mass spectrometry (IMS) (Yang *et al.*, 2009, Watrous & Dorrestein, 2011, Esquenazi *et al.*, 2009) and live colony NanoDESI mass spectrometry to monitor, *in situ*, changes in gene expression and production of metabolites during bacteria-protozoa interactions.

Results and Discussion

Transcriptional response of P. fluorescens SS101 - protozoa interactions

When challenged with *N. americana,* up to 2.3% of the SS101 genes exhibited significantly altered expression in cells located at the interaction interface. In total, 128 genes were differentially expressed in SS101 with 55 genes up-regulated and 73 genes down-regulated (fold-change ≥ 2.0; P value <0.05) (Figure 1A). The LP biosynthesis genes *massA*, *massB* and *massC* in SS101 were more than 2-fold up-regulated (Figure 1B). This up-regulation is consistent with qRT-PCR results obtained previously (Mazzola *et al.*, 2009). Also the massetolide-specific *luxR*-type transcriptional regulatory gene *massAR* and the downstream ABC-type efflux genes *macA* and *macB* were significantly up-regulated (Figure 1B). Several of the other differentially regulated genes (17 and 29 genes up and down, respectively) were classified as "Function unknown" or "Not in COGs" categories (Figure S1, category S and X, respectively). These results suggest that a large proportion of the bacterial genes expressed in response to *N. americana* are unknown and remain to be characterized. Thirteen out of sixteen genes from the "Amino acid transport and metabolism" category were up-regulated, including genes associated with arginine and proline metabolism, lysine biosynthesis, degradation of aromatic compounds and phenylalanine metabolism, respectively. The *yveA* gene, which mediates uptake of both l-aspartate and l-glutamate (Lorca *et al.*, 2003), was 3-fold up-regulated in SS101 in interaction with *N. americana* (Table S1). PflSS101_1522, a homologue gene of *ilvB* in *Pseudomonas protegens*, was 4-fold up-regulated in SS101 upon interaction with *N. americana*. IlvB is a large subunit of acetohydroxyacid synthase (AHAS) which catalyses the first step in the biosynthesis of the essential amino acids isoleucine, leucine and valine in bacteria, as well as in plants, fungi and certain algae (Mitra & Sarma, 2008, Nelson & Duxbury, 2008). The up-regulation of several genes involved in amino acid transport and metabolism suggests that the interaction with *N. americana* induces changes in primary metabolism of *P. fluorescens* SS101. In our previous study (de Bruijn & Raaijmakers, 2009), we found indications that amino acids affect the production of the lipopeptide massetolide A in SS101. Hence, the observed transcriptional changes in amino acid metabolism may, via LP biosynthesis, modulate defense against protozoan predation.

In SS101, the extracellular alkaline metalloprotease encoding gene *aprA* was 2.4-fold up-regulated. Although the role of AprA in defense of SS101 against protozoan predation remains to be tested, proteases are known to contribute to the defense of *P. protegens* CHA0 and *Vibrio cholerae* to protozoa (Jousset *et al.*, 2006, Vaitkevicius *et al.*, 2006, Niu *et al.*, 2010). Among the down-regulated genes were 5 genes from the role category "inorganic ion transport and metabolism" (Figure S1). Another down-regulated gene was a TetR family transcriptional regulator (PflSS101_2501). The TetR-family of transcriptional regulators (TFR) is a large and important family of one-component signal transduction systems (Ramos *et al.*, 2005). TFRs are known to interact with an

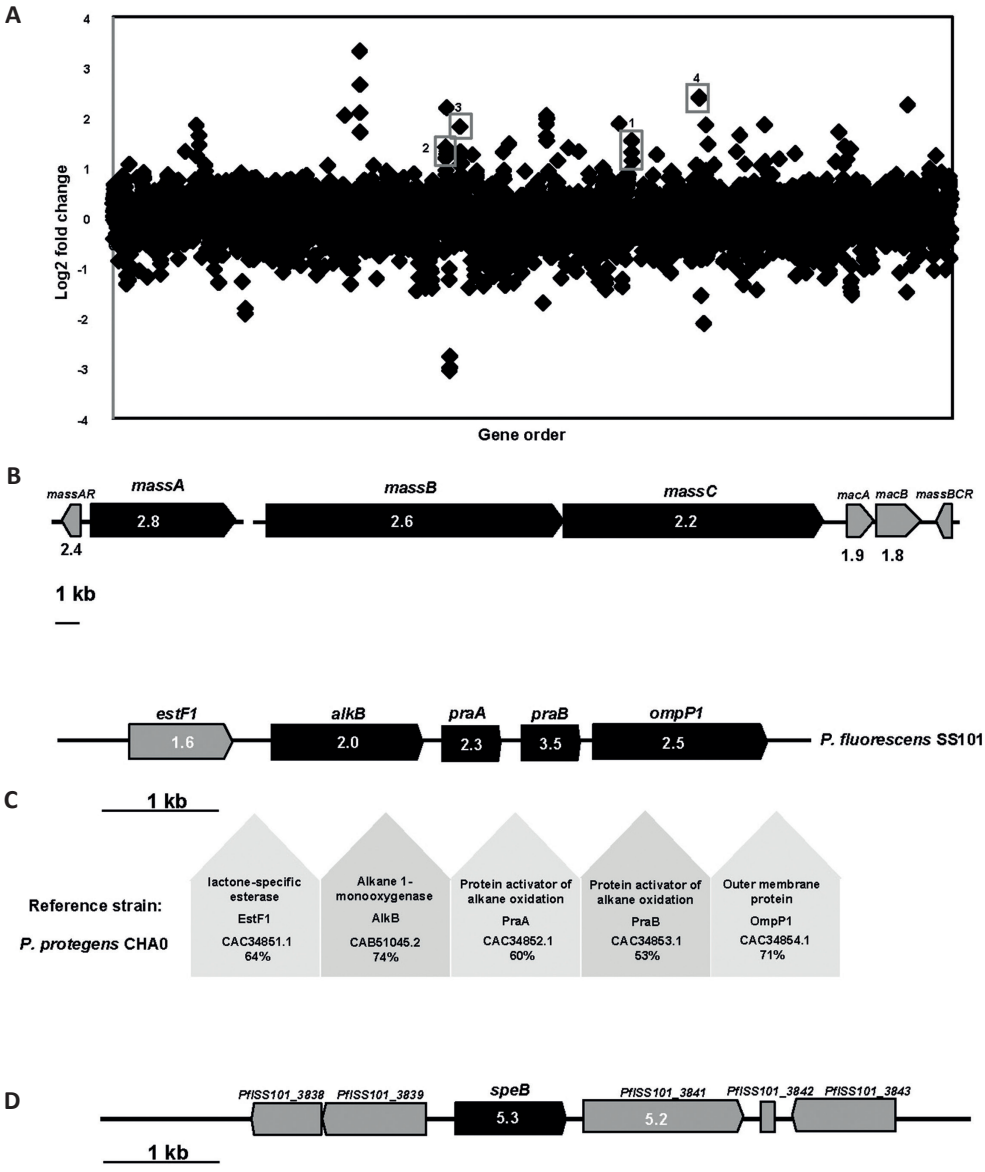

Figure 1. (A) Transcriptomic analysis of *P. fluorescens* SS101-*N. americana* interaction. Each point represents one annotated gene in the SS101 genome, with the X-axis showing the gene order, and the Y-axis showing the log2 of gene transcript abundance in the interaction. The identities of highly modulated, well-characterized gene clusters are shown. 1. *massA*; 2. *massB*, *massC*; 3. alkane oxidation gene clusters; 4. agmatinase encoding gene *speB*. (B) Organization of the lipopeptide (LP) gene cluster in *P. fluorescens* SS101. The three LP biosynthesis genes are designated *massA*, *massB* and *massC*. In the boxes of the genes are the fold changes in their expression during *P. fluorescens-N. americana* interaction. (C) Organization of the alkane oxidation gene cluster in SS101. The reference strain used is *P. protegens* CHA0 (previously described as *P. fluorescens*). In the boxes of the genes are the fold changes in their expression during *P. fluorescens-N. americana* interaction. (D) Organization of the putrescine encoding gene *speB* and its flanking genes. In the boxes of the genes are the fold changes in their expression during *P. fluorescens-N. americana* interaction.

exceptionally diverse set of small molecules, including antibiotics, metabolites, and cell-cell signalling molecules (Cuthbertson & Nodwell, 2013). For instance, the macrolide antibiotic, avermectin, produced by *S. avermitilis*, was recently shown to be negatively regulated by a TetR-family transcriptional regulator (Guo *et al.*, 2013). The function of the TFR genes in interactions between *P. fluorescens* SS101 and *N. americana* remains unknown.

Alkane oxidation/degradation genes up-regulated in bacteria - protozoa interactions
In the SS101-protozoa interaction, we observed that the gene cluster PflSS101_2280-2283 was up-regulated, with significant fold changes ranging from 2.0 to 3.5 (Figure 1C). BlastX analysis revealed that these genes are orthologues (52%-74% identities) of *alkB*, *praA*, *praB* and *ompP1* of the alkane oxidation/degradation gene cluster from *P. protegens* CHA0 (Figure 1C). AlkB encodes an integral membrane alkane hydroxylase which is essential for growth of *P. protegens* CHA0 on C12-C16 n-alkanes. Inactivation of this gene significantly reduced the capacity of CHA0 to protect plants against soil-borne diseases such as black root rot of tobacco and take-all disease of wheat (Smits, 2001). PraA and PraB are two activators of alkane oxidation and showed alkane-solubilizing effects after overexpression in *E. coli*. A *praA* mutant in *P. aeruginosa* PG201 was found to be retarded in its growth in n-hexadecane-containing media (Hardegger *et al.*, 1994). Additionally, genes involved in the alkane degradation process including alcohol dehydrogenase (PflSS101_1413, *adhB*) and aldehyde dehydrogenase (PflSS101_2843) were up-regulated in the *Pseudomonas*-protozoa interactions. Some *Pseudomonas* species employ biosurfactant-mediated solubilisation to enable use of long chain alkanes as a carbon source (Fiechter, 1992, Urs A. Ochsner, 1996). This process may function as a means to store excess carbon which can subsequently be utilized by the bacterium as an endogenous energy source during starvation periods (Rojo, 2009). Alternatively, products of the alkane oxidation could serve as precursors for the production of certain antifungal secondary metabolites, such as 2,4-DAPG (Fenton *et al.*, 1992, Keel *et al.*, 1992). Examination of such a premise and the potential link between alkane degradation and lipopeptide biosynthesis in strain SS101 is not known but would be interesting to examine in future studies.

A putrescine catalysis encoding gene is up-regulated in bacteria - protozoa interactions
We observed that the agmatinase encoding gene *speB* (PflSS101_3840) was more than 5-fold up-regulated in the SS101-protozoa interaction (Figure 1D). In bacteria, the gene product of *speB* is responsible for catalysing the conversion of agmatine to putrescine (Nakada & Itoh, 2003, Cunin *et al.*, 1986). A transporter gene (PflSS101_3841), located adjacent to the up-regulated agmatinase gene was also up-regulated 5.2-fold in SS101 cells interacting with *N. americana* (Figure 1D). Putrescine is a polyamine known to be involved in a variety of functions. It can be utilized by bacteria as both carbon and nitrogen source and is required for optimal growth (Tabor & Tabor, 1985) and root colonization (Kuiper *et al.*, 2001). Putrescine can act as an intercellular signal for

swarming in *Proteus mirabilis* (Sturgill & Rather, 2004) and protects *Escherichia coli* cells from the toxic effects of oxygen (Chattopadhyay *et al.*, 2003). It can also restore biofilm formation of an arginine decarboxylase (SpeA) and ornithine decarboxylase (SpeC) double mutant in *Yersinia pestis* (Wortham *et al.*, 2010). These findings suggest that putrescine may provide protection, directly or indirectly, to strain SS101 against predation by *N. americana*.

Effects of putrescine on N. americana viability

In vitro assays were conducted to examine the effect of putrescine on trophozoites of *N. americana*. The results of dose-dependent experiments showed that putrescine induced trophozoite encystment (Figure 2A). The time required for induction of trophozoite encystment decreased with increasing putrescine concentrations. At a putrescine concentration of 50 mM, all trophozoites encysted within approximately 10 min whereas at a concentration of 250 mM or higher, the time to encystment was approximately 1.5 min. From a concentration of 350 mM onward, there were no observable cysts. This was likely due to trophozoite lysis, which in some instances left visible remnants of deflated trophozoites. Already after 7 seconds exposure to 250 mM putrescine, trophozoites started to deflate (Figure 2B). Subsequently, cyst viability was assessed by determining the average number of trophozoites obtained from putrescine-treated cysts transferred to the surface of water agar plates amended with heat-killed *E. coli*. Cyst viability decreased with prior exposure to increasing concentrations of putrescine (Table 1).

Table 1. Average number of trophozoites yielded after transferring putrescine-treated cysts to water agar plates with PAS and heat-killed *E. coli*

Putrescine concentration (mM)	Cysts/µl	Trophozoites/µl
0 (Control)	72.13	79.93
50	18.33	54.17
100	10.00	5.00
150	15.55	4.49
200	25.98	2.64
250	15.41	0.61
300	18.32	0.31
350	14.44	0.09
400	12.54	0.09
450	11.18	0.02
500	14.52	0.01

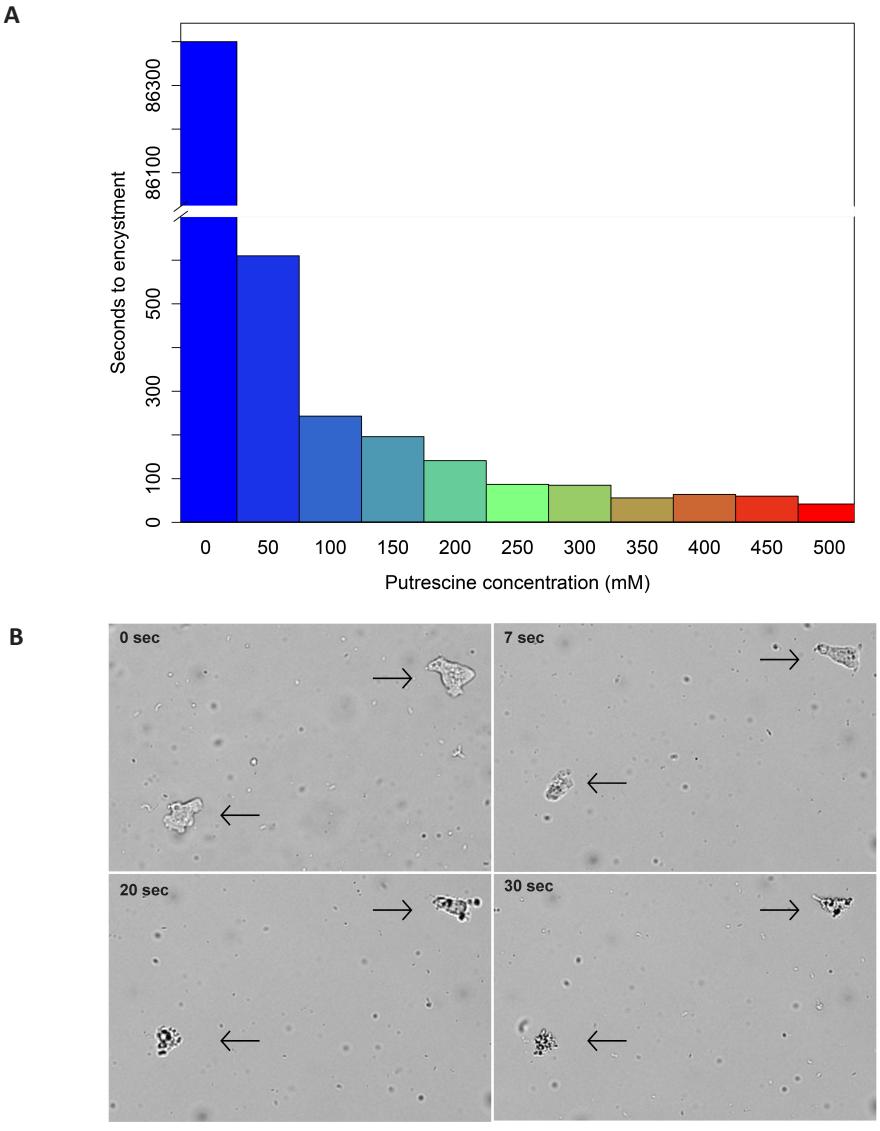

Figure 2. (A) Time to encystment of amoeboid and flagellate forms of *N. americana* exposed to increasing concentrations of putrescine. (B) Trophozoite viability at 0, 7, 20 and 30 seconds after exposure to 250 mM putrescine.

Metabolites produced in Pseudomonas-protozoa interactions

Similar to the experimental set-up used for the transcriptional profiling, strain SS101 was streaked across the surface of solid 0.2 X NBY medium using an inoculation loop (Figure 3A). After 3 h incubation at 25°C, 5 µL of a suspension containing 200 *N. americana* cysts μL^{-1} was spotted at one end of the linear bacterial growth, and the plates were incubated at 25°C for 3 days. A section of the agar containing the interaction zone was applied to

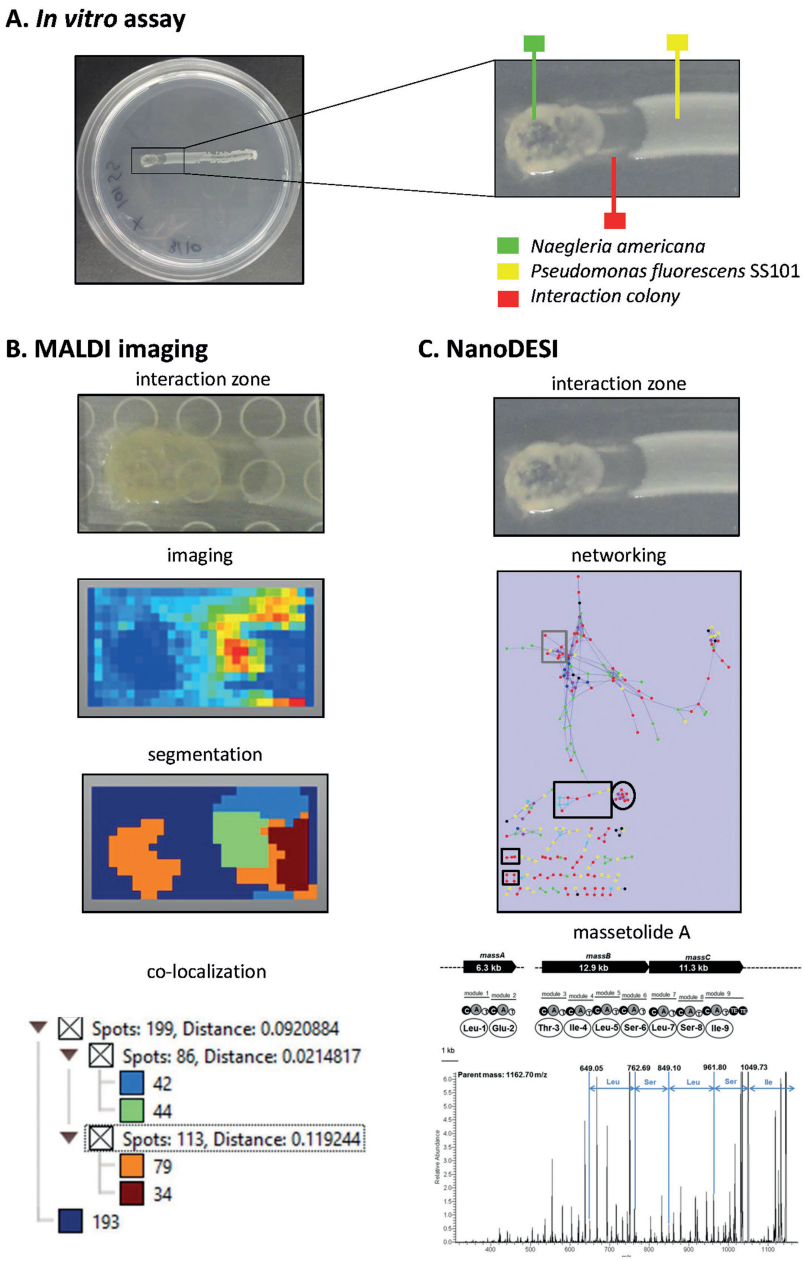

Figure 3. Experimental setup of MALDI imaging mass spectrometry (IMS) analysis of *Pseudomonas*-protozoa interactions. (A) Spatial segmentation and cluster tree of the *P. fluorescens* SS101-*N. americana* MALDI IMS data. (B) The MS/MS network and annotation of ion clusters from *P. fluorescens* SS101-*N. americana* interaction. (C) Black square designates the lipopeptide massetolide A and its derivatives; Black circle defines the 325-477 m/z ion cluster; Grey square specifies the 766-796 m/z ion cluster. MS/MS analysis indicated that the parent ion 1162.70 m/z detected during *P. fluorescens SS101- N. americana* is most likely massetolide A. Complete lists of the ion clusters from (C) are given in Tables S4, S5 and S6, respectively.

MALDI-TOF to study the secreted metabolites by IMS (Figure 3B). In addition, live colony NanoDESI mass spectrometry was performed on the protozoan colony, the interaction zone and the *Pseudomonas* colony to construct MS/MS metabolite networks. Nodes with a high MS/MS spectral analogy cluster together and often belong to the same chemical class (Watrous *et al.*, 2012). Clusters of the different metabolite classes were then compared to the ions observed in the MALDI IMS data. We detected metabolites produced by *Pseudomonas* alone (yellow nodes), protozoa alone (green nodes) and produced during the *Pseudomonas*-protozoa interaction (red nodes). The network was constructed combing the different samples per species together (Figure 3C).

Metabolite classes in P. fluorescens SS101 - N. americana interaction

Spatial segmentation analysis of the MALDI IMS data revealed four specific classes of metabolites indicated with different colours (light blue, green, orange and dark red), that were co-localized in the *Pseudomonas*-protozoa interaction zone (Figure 3B; Table S2). There were 6 and 14 ions found to be co-localized within the light blue and green cluster, respectively, with correlation values greater than 0.5 (Figure 3B; Table S2) including 8 ions with predicted masses ranging from 1136 m/z to 1201 m/z (Figure 4A). These ions were not detected in the massetolide-deficient Δ*massA* mutant or *N. americana* alone (Figure 4A). The absence of these ions in the Δ*massA* mutant suggests that they are massetolide derivatives. Box plots further confirmed that the intensities of these ions were higher in wild type strain SS101, and in the SS101-protozoa interaction than in the samples with *N. americana* or Δ*massA* alone (Figure 4B). Masses of massetolide A and its derivatives also clustered together in the MS/MS network (Figure 3C; Table S4). Tandem MS of the ion with a mass of 1163 m/z indicated a peptide sequence of leucine, serine, leucine, serine and isoleucine. These amino acids are identical to the C-terminal peptide sequence of massetolide A (Figure 3C). Based on our previous study, the mass of massetolide A is 1140 and the masses of its derivatives range from 1112-1158 m/z (de Bruijn *et al.*, 2008). The larger ion masses detected here are most likely due to a sodium (molecular weight: 22.989) gain during ionization. Although the intensities of the massetolides were not different between SS101 and SS101-protozoa interaction (Figure 4B), we observed a striking difference in spatial distribution of massetolide A. In absence of the protozoa, the lipopeptide was more homogeneously distributed in the SS101 colony, whereas in presence of protozoa it localized predominantly in the bacterial cells at the interaction zone (Figure 4A). To our knowledge, this is the first report of the real time visualization and spatial distribution of LPs during bacteria-protozoa interactions.

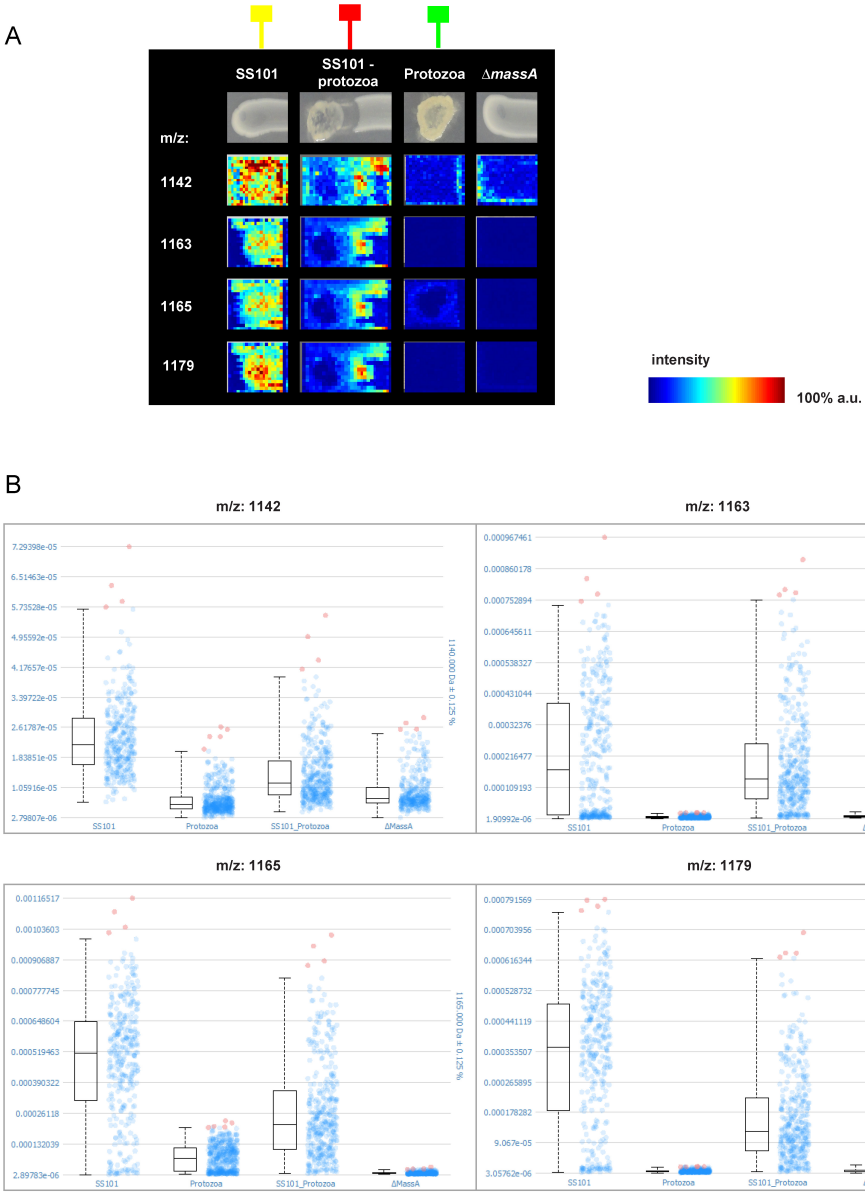

Figure 4. (A) MALDI imaging mass spectrometry (IMS) shows production of massetolide A and its derivatives during the *P. fluorescens* SS101-*N. americana* interaction. a.u. = arbitrary units. (B) Box plots depicting the production of massetolide A and its derivatives in *P. fluorescens* SS101 alone, *N. americana* alone, *P. fluorescens* SS101-*N. americana* interaction and *massA* mutant alone.

Apart from the massetolides, other ions with predicted masses ranging from 249-688 m/z were co-localized with the green cluster in the segmentation map (Figure 3B, Table S2). These ions were detected in the interaction, in bacteria or in the protozoa alone (Figure 5A; Table S2). One of the ions with a mass of 477 m/z clustered with seven other ions in the MS/MS network (Figure 3C; Table S5). Ion 325 m/z, whose intensity is higher in the bacteria-protozoa interaction than in the bacteria alone (Figure 5A), will need to be investigated in more detail by tandem MS analyses to resolve its identity. Furthermore, next to the representative ions shown in Figure 5B, a number of other ions were present (Table S2) including three ions with a mass of 740 m/z, 741 m/z and 767 m/z belonging to the green class, and sixteen ions with masses ranging from 703 m/z to 790 m/z (Table S2) belonging to the dark red class from the segmentation map. The ion with a mass of 766 m/z clustered in the network with nine other ions with masses ranging from 752 m/z to 796 m/z (Figure 3C; Table S6). Preliminary MS/MS analyses showed that four of these ions exhibit a similar fragmentation pattern with a 123.9 Da loss (not shown). Resolving the identity of this metabolite class will be subject of future experiments.

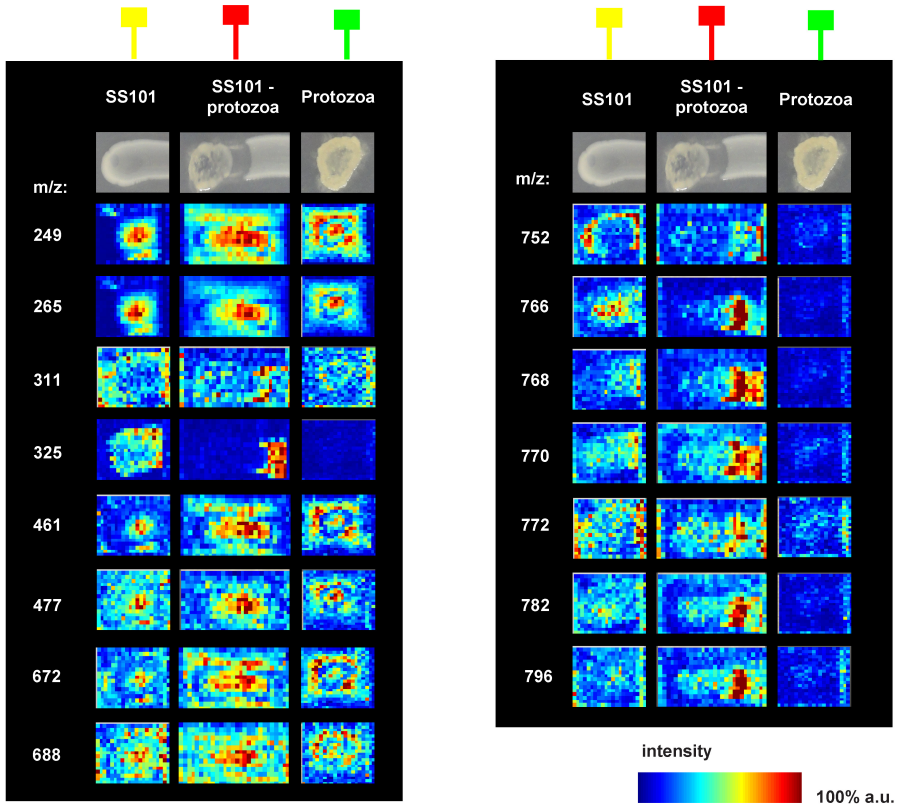

Figure 5. (A) MALDI imaging mass spectrometry (IMS) shows production of 249-688 m/z ions in the MS/MS network of the *P. fluorescens* SS101-*N. americana* interaction. a.u. = arbitrary units. (B) MALDI imaging mass spectrometry (IMS) shows production of 752-796 m/z ions cluster in the MS/MS network of the *P. fluorescens* SS101-*N. americana* interaction.

General and strain-specific responses to protozoan predation

To determine if the observed transcriptional and metabolic responses are specific for strain SS101 or more generally found in *P. fluorescens*, we conducted similar studies employing *P. fluorescens* SBW25. At the transcriptional level, 135 genes showed differential expression in SBW25, with 65 genes up-regulated and 70 genes down-regulated (fold-change $\geq$ 2.0; *P* value <0.05). Twenty five genes in SBW25 exposed to protozoa showed a similar transcriptional response as their orthologues in strain SS101 (Figure S3, Table S3). The 17 up-regulated genes included the viscosin biosynthesis genes, the alkane degradation gene cluster and also *speB*, the agmatinase gene involved in putrescine biosynthesis. At the metabolic level, viscosin production in SBW25-protozoa interaction was confirmed (Figure S4). Similar to that observed for massetolide produced by SS101, also the lipopeptide viscosin was localized at the interaction site between SBW25 and *N. americana*, whereas in the absence of protozoa the viscosin was more homogeneously distributed in the bacterial colony. This indicates that site-directed lipopeptide production is a general defense mechanism for at least two *Pseudomonas fluorescens* strains when confronted with protozoa.

A specific ion was detected with a mass of 88.66 and 88.348 m/z by MALDI IMS for SS101 and SBW25 respectively (Figure S5). The theoretical mass of protonated putrescine ion species is $[M+H]^+$ 89.1 amu. These data suggest that the detected ion is putrescine, further supporting the transcriptome data for *P. fluorescens* SS101 and SBW25 (Figure S3). Similar to SS101, production of ions with masses of 325 m/z and 766 m/z were detected in the SBW25-protozoa interaction (Figure S6). Comparisons of the MS/MS profiles with the ones found in SS101-protozoa interaction indicated that these represent the same metabolite class(es) produced in the interactions for both strains. Strain-specific metabolites were also observed in the SBW25-protozoa interaction (Figure S7, S8) and the identities of these metabolites are currently under investigation.

Conclusions

Whole genome transcriptomic analysis of *Pseudomonas fluorescens* SS101 in confrontation with the protozoan predator *N. americana* revealed altered expression for 2.3% of genes from the SS101 genome. Among these changes, lipopeptide biosynthesis genes, together with the adjacent transcriptional regulator were up-regulated, which extended our initial findings of the role of lipopeptides as an anti-predation defense mechanism (Mazzola *et al.*, 2009). Moreover, we showed that putrescine biosynthesis in SS101 was up-regulated in response to challenge by *N. americana*. Subsequent experiments revealed, for the first time, that putrescine induces protozoan trophozoite encystment and affects cyst viability. Subsequent to the transcriptomic analysis, metabolic analysis of this interaction was conducted via MALDI imaging mass spectrometry (IMS) and live colony NanoDESI mass spectrometry. To date, most information on these techniques focuses on microbes alone (Watrous *et*

al., 2012), bacteria-bacteria or bacteria-fungi interactions (Moree *et al.*, 2013, Traxler *et al.*, 2013, Moree *et al.*, 2012). Here, new information is provided on the chemistry of bacteria-protozoa interactions. Our study identified, for the first time, real time and site-specific lipopeptide production at the interface of *Pseudomonas*-protozoa interactions and demonstrated that closely related bacterial strains exhibit common and unique transcriptomic and metabolic responses to predation.

Material and Methods

Protozoa, bacteria and growth conditions. *Pseudomonas fluorescens* strains SS101 and SBW25 were grown on Pseudomonas agar F (Difco) plates or in liquid King's medium B (KB) at 25°C. *Escherichia coli* was grown on Luria-Bertani (LB) plates or in LB broth. The amoeba-flagellate *Naeglaria americana* was used as the protozoan predator. The protist was propagated by cultivation with heat-killed *E. coli* DH5α as the food source. 5 µL of bacterial cells ($\sim 10^8$ cells) were added to a water agar surface contained in a 9-cm-diameter petri plate, and was subsequently overlaid with 3 ml of Page's modified Neff's amoeba saline (PAS) solution (Rowbotham, 1983). The plates were then inoculated with 200 µl of a *N. americana* cyst suspension (200 cysts μl^{-1}), sealed with Parafilm, and incubated at 20°C with 2 ml PAS added to the plates at 7-day intervals.

***Pseudomonas-N. americana* interaction assay**

Bacterial strains pre-cultured on KB agar were streaked across the surface of 0.2 X nutrient broth-yeast extract (NBY) (1 Liter contains 1.6 g nutrient broth, 0.4 g yeast extract, 1.0 g glucose, 15 g agar) at a width of 4 mm using a transfer loop. After 3 hours incubation at 25°C, 5 **µl** of a suspension containing 200 *N. americana* cysts μl^{-1} was spot-inoculated at one end of the linear bacterial growth, and the plates were incubated at 25°C for 3 days. Bacterial cells were collected from the zone of interaction using a transfer loop with 3 replicates for each strain.

The effect of putrescine on encystment and viability of *N. americana*

Putrescine was added to an aqueous environment to *N. americana* trophozoites to final concentrations of 50-500 mM. Encystment of the trophozoites when exposed to putrescine was determined microscopically. For each putrescine concentration, four replicates were used.

To determine viability of the *N. americana* cysts, putrescine-treated cysts were transferred to water agar plates with PAS and heat-killed *E. coli*. The average number of trophozoites emerging from the cysts was determined microscopically after 24 hrs of incubation at 24°C. Microscopic photos with 100X magnification were taken after 0, 7, 20 and 30 seconds of exposure to 250 mM putrescine using a Zeiss confocal microscope with transmitted light.

Transcriptional profiling

Pseudomonas fluorescens strains SS101 and SBW25 were collected from the bacteria-protozoa zone of interaction and at the corresponding location on the control plates not inoculated with *N. americana*. Total RNA was extracted from the bacterial cells with Trizol reagent (Invitrogen) and further purified with the NucleoSpin RNA kit. Four replicates were used for each bacterial strain. Tiling microarrays for *Pseudomonas fluorescens* SS101 and SBW25 were developed in the Dutch Genomics Service & Support Provider, University of Amsterdam (UvA), Amsterdam, the Netherlands. In total, 134,276/134,858 probes (60-mer) were designed with, in general, a gap of 32/46 nucleotides between adjacent probes on the same strand and an overlap by 14/7 nucleotides when regarding both strands in SS101 and SBW25, respectively. In addition, 5,000 custom negative control probes were hybridized and used as an internal control to validate and normalize the designed probes in a CGH experiment of 4 arrays. Probes were annotated and assembled into probe sets for known genes based on location information retrieved from the Pathosystems Resource Integration Center (PATRIC, http://patricbrc.org). Probes outside of known genes were labelled as InterGenic Region (IGR). cDNA labelling was conducted as described previously (de Knegt *et al.*, 2013). Briefly, cDNA was synthesized in the presence of Cy3-dUTP (Cy3) for the test samples and with Cy5-dUTP (Cy5) for the common reference. The common reference was made by an equimolar pool of the test samples (3 µg per sample). 5 µg of total RNA per reaction was used and yielded 1.5-2.5 µg cDNA for each sample with larger than 16 pmol of Cy3 or Cy5 dye per microgram.

Hybridizations were performed according to Pennings et al. (2011). Slides were washed according to the procedures described in the Nimblegen Arrays User's Guide - Gene Expression Arrays Version 5.0 and scanned in an ozone-free room with an Agilent DNA microarray scanner G2565CA (Agilent Technologies). Feature extraction was performed with NimbleScan v2.5 (Roche Nimblegen). Data pre-processing consisted of $\log_2$-transformation of the raw probe-intensity data, followed by a within slide Lowess normalization. Thus normalized sample (Cy3) channel intensities were summarized into probe set values and normalized between arrays using the RMA (Robust Multi-Array Analysis) algorithm (Irizarry, et al. 2003). All results described were found to be significant using a false discovery rate of less than 5%. Analysis of the gene expression data was conducted by Arraystar software. Microarray data were validated by quantitative PCR experiments for several genes (data not shown).

MALDI-imaging mass spectrometry (IMS) and live colony mass spectrometry (NanoDESI) of *Pseudomonas-N. americana* interactions

Pseudomonas strains SS101, SBW25 and their lipopeptide deficient *massA* and *viscA* mutants, respectively, were streaked across the surface of solid 0.2 X NBY medium at a length of 4-cm using an inoculation loop. After 3 h incubation at 25°C, 5 µL of a suspension containing 200 *N. americana* cysts μL^{-1} was spotted at one end of the linear bacterial growth, and the plates were incubated at 25°C for 3 days. Thin layer interaction

agar plates of *Pseudomonas* and *N. americana* were prepared and then sprayed with Universal MALDI matrix (Sigma-Aldrich). MALDI-imaging of the interaction samples on a Bruker MSP 96 anchor plate was performed on a Microflex Bruker Daltonics mass spectrometer outfitted with Compass 1.2 software suite (Watrous *et al.*, 2012). To detect metabolites produced in the interaction zone, *Pseudomonas-N. americana* interaction plates were used to perform live colony mass spectrometry using NanoDESI as described previously (Watrous *et al.*, 2012).

SCiLS Lab analysis of MALDI-imaging mass spectrometry (IMS) data

The software SCiLS Lab version 2014b (SCiLS, Bremen, Germany) was used for MALDI-IMS data analysis to detect ions that have higher abundance at a specific condition, i.e. the bacteria-protozoa interaction. Raw data from *P. fluorescens* SS101, *massA* mutant, *P. fluorescens* SBW25, *viscA* mutant, *N. americana* alone, and *N. americana* interacting with each of the bacterial strains/mutants were imported individually. The individual datasets were then grouped to allow for the comparisons. In total, the complete data set was comprised of 4126 spectra each with 190454 datapoints in the mass range of 0-5 kDa. The data was processed using the Preprocessing Pipeline of SCiLS Lab 2014b using the default settings. This includes baseline reduction using iterative convolution with 20 interactions and sigma set to 20 and normalization to the total ion count (TIC). Automatic spatial segmentation was used as a first step in data mining. In this approach, similarities between spectra were statistically determined and similar spectra were grouped into a cluster. All spectra within a particular cluster were assigned a selected colour and displayed as a spatial segmentation map in which pixels were colour-coded according to their cluster assignment. For each cluster, its spatial region was considered and co-localized ion images were found as correlated to the region with the Pearson correlation of 0.5 or higher; the m/z-values of co-localized ions are listed in Table S2. Individual *m/z* images were created from the selected ions with a hotspot removal applied for better visualization. In order to compare the intensity of ions of interest in different samples, single *m/z* values were also displayed in intensity box plot. The low and high quantiles for the hotspot removal and the intensity box plot were set to 0.00% and 99.00%, correspondingly.

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Supplementary data

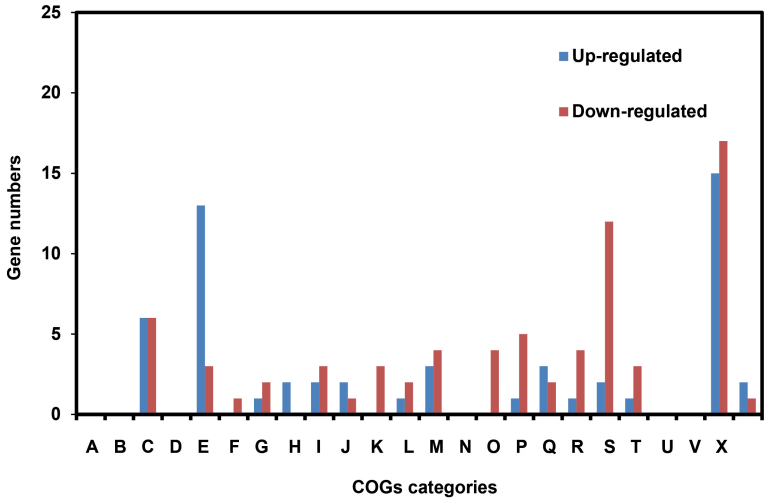


Figure S1. The number of genes that are up-regulated (Blue) or down-regulated (Red) in *P. fluorescens* SS101 cells exposed to *N. americana.* The genes are categorized into COGs A thru X (for specification of each of the COGs, see Table S1). Some genes can be placed in more than one COG and thus counted more than once.

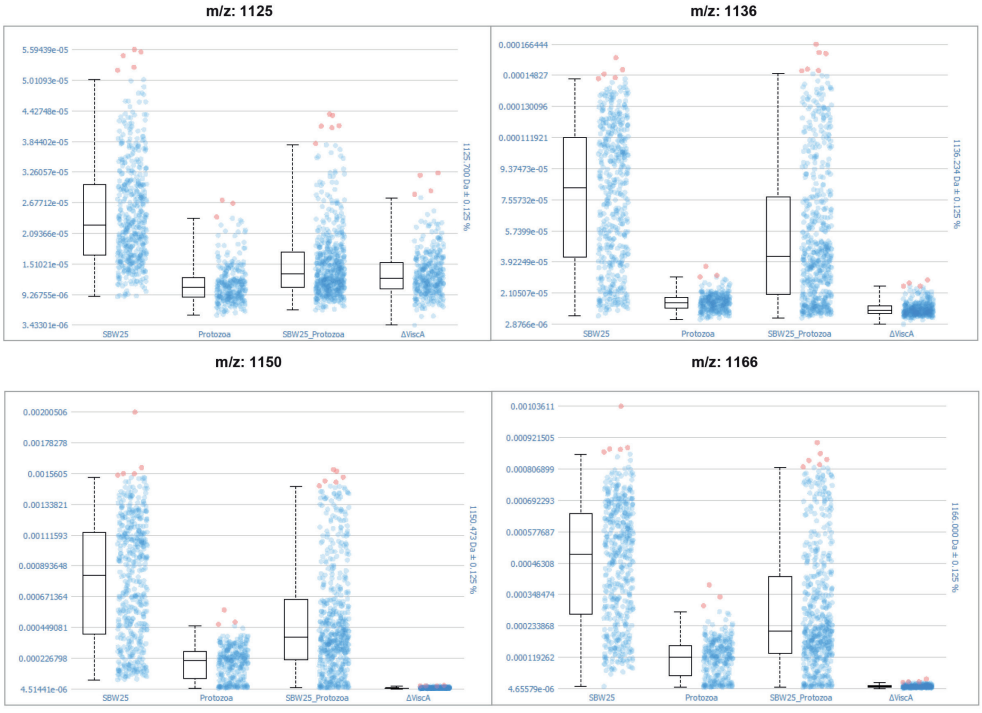


Figure S2. Box plots depicting the production of viscosin and its derivatives in *P. fluorescens* SBW25 alone, *N. americana* alone, *P. fluorescens* SBW25-*N. americana* interaction and *viscA* mutant alone.

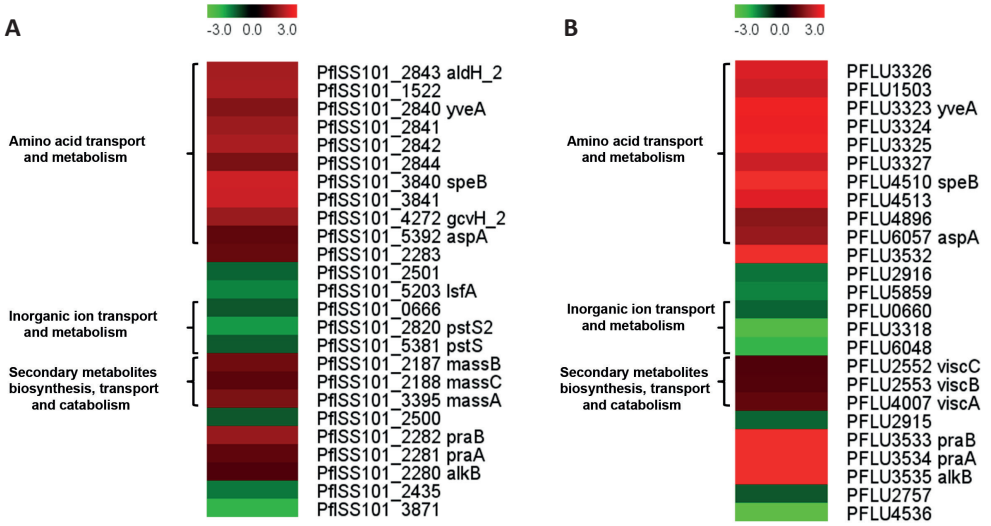
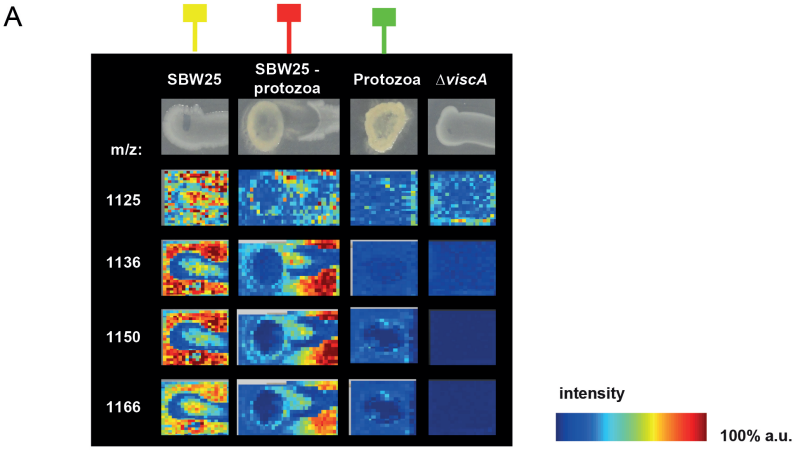
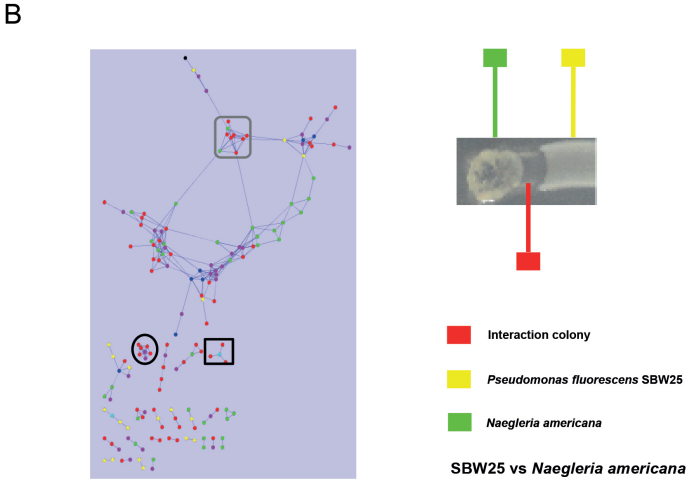


Figure S3. Whole genome transcriptome analysis of *P. fluorescens* strains SS101 and SBW25 in the presence of *N. americana*. Heat maps showing $\log_2$-fold changes in the expression of genes that are differentially regulated in both SS101 (A) and SBW25 (B) upon protozoan grazing. Wild type SS101 and SBW25 were grown on KB plates at 25ºC in the presence of *N. americana* for 2-3 days. Cells were collected and total RNA was extracted followed by cDNA synthesis, labelling and hybridization to a SS101/SBW25 whole-genome tiling microarray. The fold changes shown here represent averages of four biological replicates. For a list of all genes differentially regulated in SS101 and SBW25, we refer to Supplementary Tables S1 and S3.





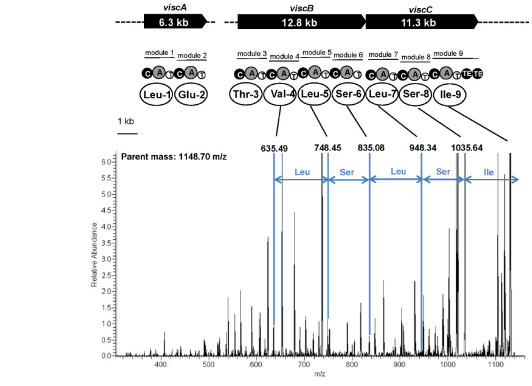


Figure S4. (A) MALDI imaging mass spectrometry (IMS) shows production of viscosin and its derivatives during the *P. fluorescens* SBW25-*N. americana* interaction. a.u. = arbitrary units. (B) MS/MS network and annotation of ion clusters from the *P. fluorescens* SBW25-*N. americana* interaction: Black square stands for viscosin and its derivatives; Black circle stands for the 325 m/z ion cluster; Grey square stands for the 766 m/z ion cluster. MS/MS analysis indicated that the parent ion 1148.70 m/z detected during *P. fluorescens SBW25- N. americana* is most likely viscosin.

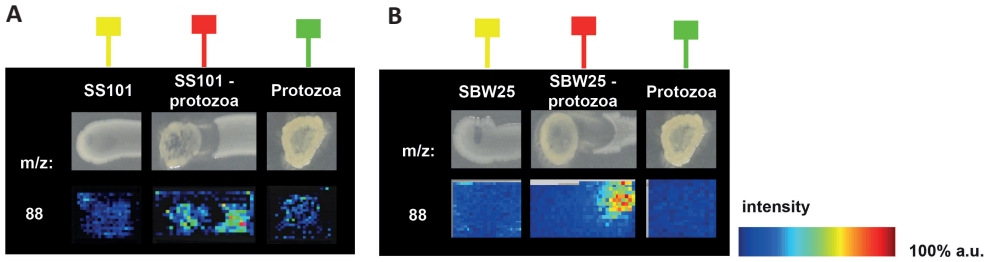


Figure S5. MALDI imaging mass spectrometry (IMS) shows production of 88 m/z ions in the *P. fluorescens* SS101-*N. americana* (A) and *P. fluorescens* SBW25-*N. americana* (B) interactions respectively. a.u. = arbitrary units.

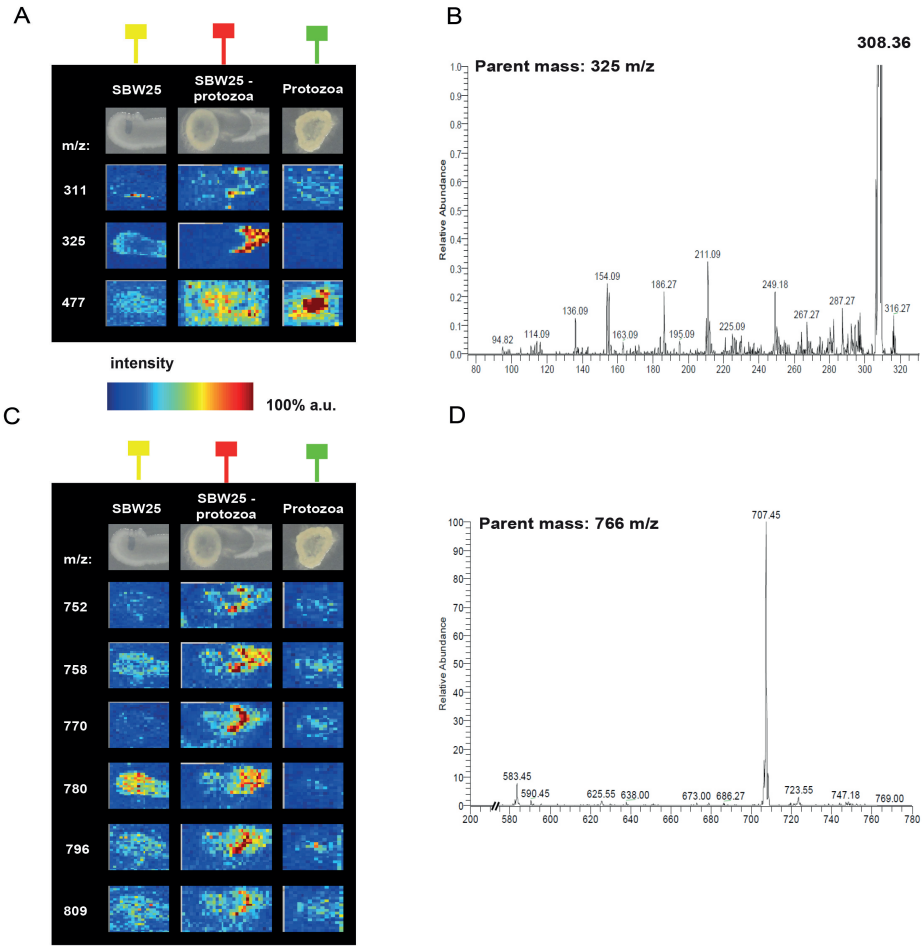


Figure S6. (A) MALDI imaging mass spectrometry (IMS) shows production of 311-477 m/z ions and its cluster ions in the MS/MS network in *P. fluorescens* SBW25-*N. americana* interaction. a.u. = arbitrary units. (B) MS/MS profile of 325 m/z during the *P. fluorescens* SBW25-*N. americana.* (C) MALDI imaging mass spectrometry (IMS) shows production of 752-809 m/z ions and its cluster ions in the MS/MS network in *P. fluorescens* SBW25-*N. americana* interaction. a.u. = arbitrary units. (D) MS/MS profile of 766 m/z during the *P. fluorescens* SBW25-*N. americana*

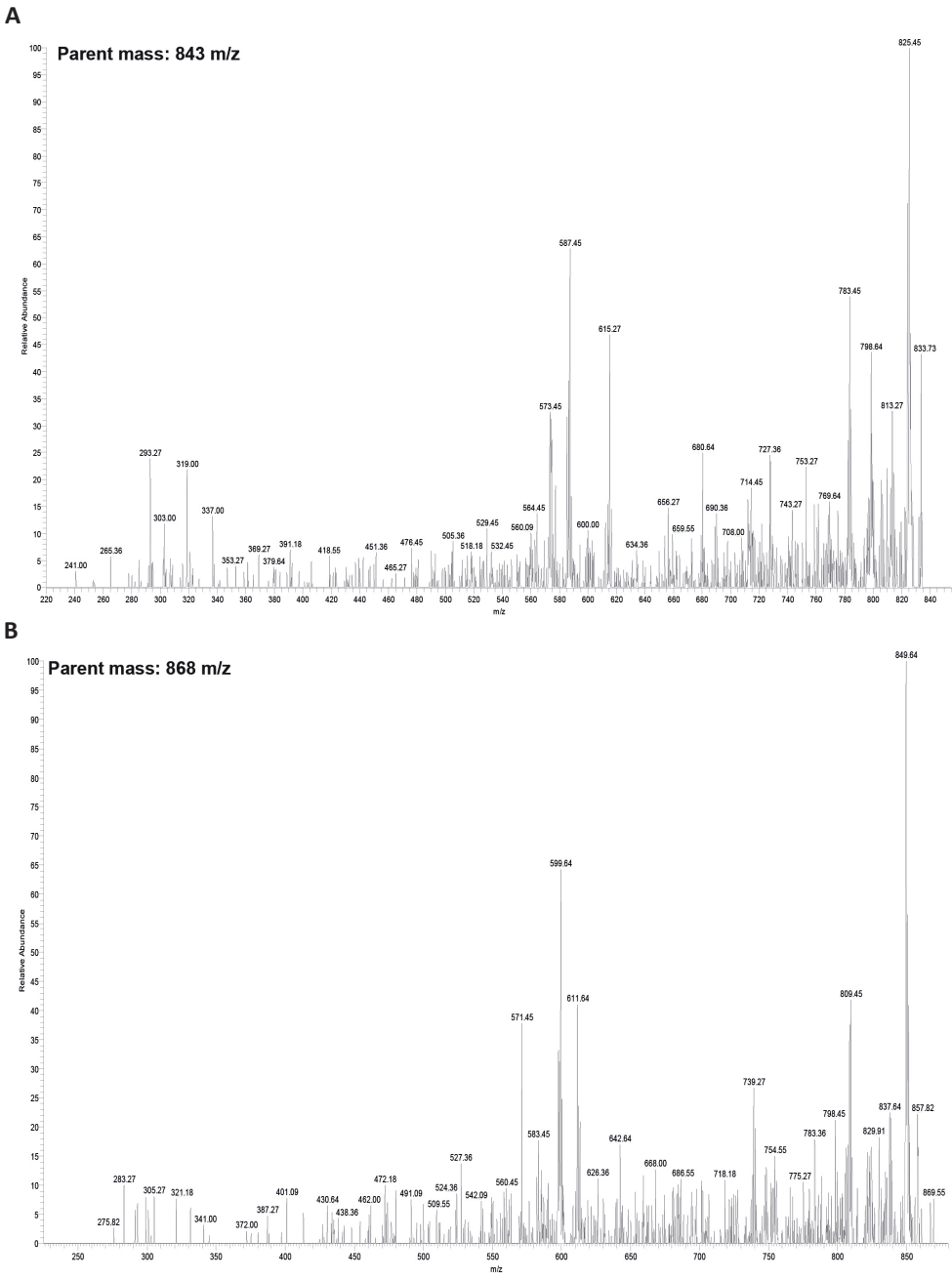


Figure S7. MS/MS profile of 843 m/z (A) and 868 m/z (B) during the *P. fluorescens* SBW25-*N. americana* interaction.

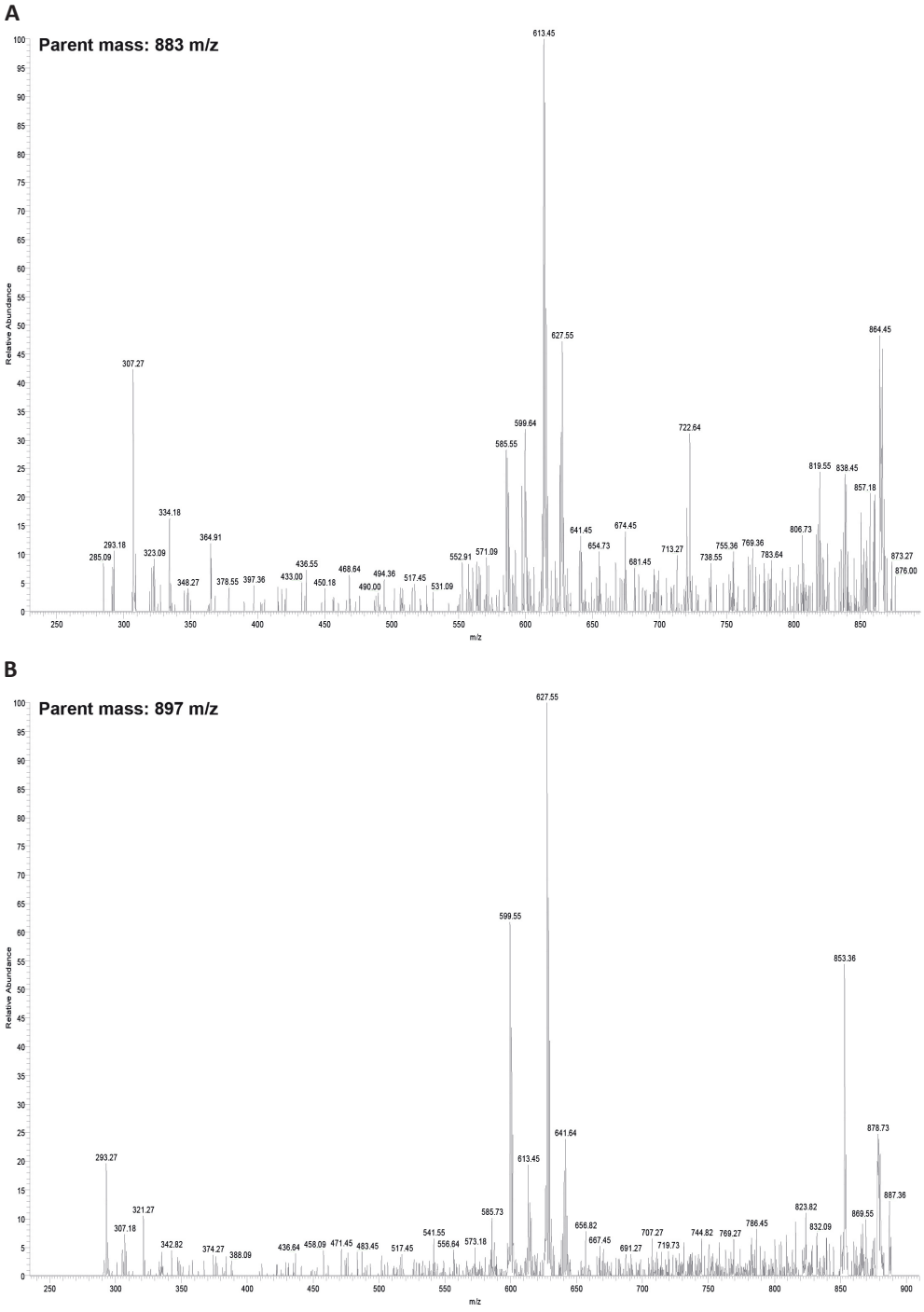
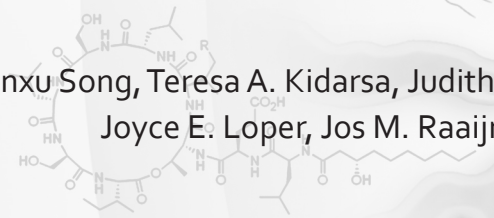


Figure S8. MS/MS profile of 883 m/z (A) and 897 m/z (B) during the *P. fluorescens* SBW25-*N. americana* interaction.

Due to the large size, supplementary tables are not shown here, but they are available upon request.

Chapter 6

Living on the edge: spatial heterogeneity and convergent evolution of social cheaters during swarming of *Pseudomonas*



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This chapter was submitted for publication

Abstract

Swarming motility is a flagella-driven multicellular behavior that allows bacteria to colonize new niches and escape competition. Here, we investigated the spatial distribution and evolution of 'social cheaters' in swarming colonies of *Pseudomonas protegens* Pf-5. Lipopeptide surfactants in the orfamide family are produced by Pf-5 and essential for swarming motility. Two orfamide-deficient mutants, with deletions in the biosynthesis gene *ofaA* or in the regulatory gene *gacA*, cannot swarm on their own but 'hitch-hiked' with wildtype Pf-5. Both mutants typify social cheaters with respect to swarming motility but exhibit distinctly different spatial distributions in co-swarming colonies, with the *ofaA* mutant moving behind the wildtype and the *gacA* mutant predominating on the edge. Experimental evolution assays showed that repeated rounds of swarming by wildtype Pf-5 drives parallel evolution toward accumulation of *gacS/gacA* spontaneous mutants on the swarming edge. The emergence of these cheaters is context dependent as they were not detected under non-swarming conditions. Results further showed that swarming colonies collapsed with increasing frequencies of *gacA* mutants. Subsequent whole-genome transcriptome analyses revealed that genes associated with resource acquisition, motility, chemotaxis and efflux were significantly upregulated in *gacA* mutants. Moreover, *gacA* mutant cells were longer and more flagellated than wildtype and *ofaA* mutant cells, which may explain their predominance on the edge of co-swarming colonies. We postulate that adaptive convergent evolution through point mutations is a common feature of range-expanding microbial populations and that the putative fitness benefits of these mutations during dispersal of bacteria into new territories are frequency-dependent.

Introduction

In natural environments, bacteria live in intimate associations with other organisms and their behaviour reflects the collective action of assemblages. Communal living and multicellular behavior are now recognized as dominant bacterial life-styles in natural environments, involving complex patterns of communication and cooperation (West *et al.*, 2007). A well-known example of cooperative behaviour of bacterial populations is swarming motility, which is defined as flagella-driven dispersal over a surface (Kearns, 2010). The intriguing and complex phenomenon of swarming motility was first recognized in *Proteus* species more than a century ago (Rather, 2005, Williams & Schwarzhoff, 1978) and has since been described for various other bacterial genera(Fraser & Hughes, 1999, Harshey, 2003, Broek & Vanderleyden, 1995, Zusman *et al.*, 2007). For several genera, swarming requires cell-to-cell contact and differentiation of vegetative cells into specialized swarmer cells (Henrichsen, 1972, Harshey, 1994, Harshey, 2003). Moisture, viscosity, surface tension, nutrients and temperature are important factors that affect swarming (Daniels *et al.*, 2004, Daniels *et al.*, 2006, Fraser & Hughes, 1999, Harshey, 2003). Swarming helps bacteria to disperse, colonize new niches (Harshey, 2003, Kearns, 2010) and to resist engulfment by bacteriophages (Ammendola *et al.*, 1998). Moreover, swarming is also linked to virulence as was shown for *Proteus mirabilis* (Allison *et al.*, 1992, Allison *et al.*, 1994).

Given that swarming is a multicellular behavior, it provides a good model to test current concepts in social evolutionary theory and to evaluate the importance of cooperation and conflict within and among bacterial populations (Diggle *et al.*, 2007, Rainey & Rainey, 2003, Rainey, 2007, Velicer & Yu, 2003, West *et al.*, 2007, van Ditmarsch & Xavier, 2014). A common form of social behavior in bacteria involves the production of so-called 'public goods', which are products produced by an individual that can be utilized by the individual itself and by its neighbors, and therefore benefit both producing and non-producing cells (West *et al.*, 2007). Public goods may have direct and indirect fitness benefits, but their production can be metabolically costly and may promote the proliferation of social cheats, i.e. cells that no longer produce the public goods themselves but instead take advantage of other producing cells in the group in a context- and frequency-dependent manner (Rainey & Rainey, 2003, Rainey, 2007, Santorelli *et al.*, 2008, West *et al.*, 2007, Ghoul *et al.*, 2014a, Ghoul *et al.*, 2014b). Bacteria secrete an array of compounds that function as public goods, including quorum sensing molecules, siderophores, exoenzymes and biosurfactants (West *et al.*, 2007, Diggle *et al.*, 2007, Griffin *et al.*, 2004, Sandoz *et al.*, 2007). Some public goods, such as rhamnolipids of *Pseudomonas aeruginosa,* play a role in the cooperative behavior underlying swarming motility (Venturi *et al.*, 2010, Xavier *et al.*, 2011).

Few studies have examined the occurrence and frequency of specific spontaneous mutations that accumulate in bacterial populations under swarming conditions and that

may affect cooperative behavior (Gardel & Mekalanos, 1996, Velicer & Yu, 2003, van Ditmarsch *et al.*, 2013). The objectives of our study were to investigate the frequency, spatial distribution and evolution of spontaneous mutants in swarming colonies of the soil bacterium *Pseudomonas protegens* Pf-5 (Howell & Stipanovic, 1979, Paulsen *et al.*, 2005). Strain Pf-5 does not have a prototypic quorum sensing system involving the production of *N*-acyl homoserine lactones, but produces a large spectrum of secondary metabolites (Gross & Loper, 2009, Loper & Gross, 2007) and two siderophores (enantio-pyochelin and a pyoverdine) (Hartney *et al.*, 2013, Youard *et al.*, 2007) that are secreted from the cell and could therefore function as public goods. These exoproducts are produced under the control of the GacS/GacA two-component system (Hassan *et al.*, 2010), which regulates the expression of many genes through a complex signal transduction pathway involving regulatory RNAs and translational repression (Lapouge *et al.*, 2008)2008. Among the numerous GacS/GacA-regulated exoproducts produced by strain Pf-5, the lipopeptide biosurfactant orfamide is known to be essential for swarming motility (Gross *et al.*, 2007a). We report that two orfamide-deficient mutants of Pf-5, with deletions in the orfamide biosynthesis gene *ofaA* or in the transcriptional regulatory gene *gacA*, 'hitch-hike' with their parental strain under swarming conditions. Both *ofaA* and *gacA* mutants typify social cheaters with respect to swarming motility but the two mutants exhibit a distinctly different spatial distribution, with the *gacA* mutant predominating on the edge of the co-swarming colonies. We conducted experimental evolution assays with wildtype Pf-5 to determine the frequency and spatial distribution of social cheaters that accumulate spontaneously in swarming colonies of Pf-5. The vast majority of these social cheaters had mutations inactivating the GacS/GacA two component regulatory system. Genetic, phenotypic, microscopic and whole-genome transcriptomic analyses were conducted to assess the fitness benefits of social cheaters that arise spontaneously during successive swarming.

Results and discussion

Co-swarming of wildtype Pf-5 and orfamide-deficient mutants

In *Pseudomonas protegens* strain Pf-5, mutations in *ofaA* or *gacA* virtually eliminated swarming motility (Fig. 1A) as described previously (Gross *et al.*, 2007a, Hassan *et al.*, 2010, Kidarsa *et al.*, 2013). Furthermore, no swarming was observed when *ofaA* and *gacA* mutants were co-inoculated with one another (Fig. 1B). When either of these mutants was co-inoculated with wildtype Pf-5, however, swarming was observed. The diameter of the swarming colony composed of the *ofaA* mutant and wildtype strain, inoculated in a 1:1 ratio, was similar to that of the swarming colony of wildtype Pf-5 alone (Fig. 1B). When the *gacA* mutant and wildtype were co-inoculated, the diameter of the swarming colony was significantly reduced compared to that of wildtype Pf-5 alone (Fig. 1B). Subsequently, we quantified the cells of Pf-5 and each of the two mutants in the co-swarming colonies based on different antibiotic resistance markers.

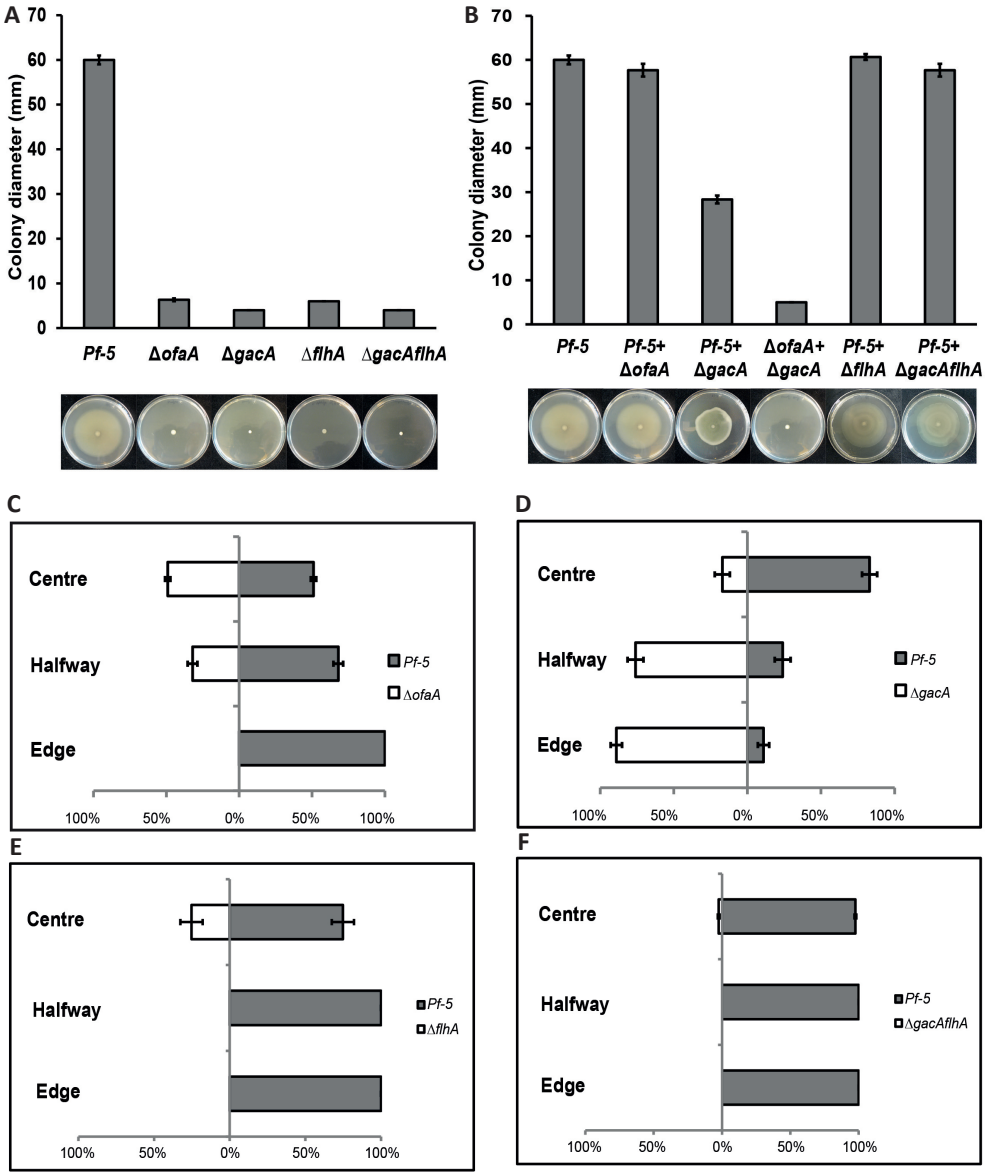

Figure 1. Swarming and co-swarming of *P. protegens* Pf-5 and mutants. (A). Swarming motility of wildtype Pf-5, Δ*ofaA*, Δ*gacA,* Δ*flhA* and Δ*gacAflhA* mutants. Cell suspensions (2µL of $10^{\wedge 8}$ cells/ml) were inoculated in the centre of soft agar (0.6% w/v) plates and incubated at 25°C. Y axis shows swarming colony diameter after 36 hrs of incubation; mean values for three replicates are given and error bars represent the standard error of the mean. (B). Swarming motility of wildtype Pf-5 alone and of mixtures of Pf-5:Δ*ofaA* (1:1), Pf-5:Δ*gacA* (1:1), Δ*ofaA*:Δ*gacA* (1:1), Pf-5:Δ*flhA* (1:1) and Pf-5:Δ*gacAflhA* (1:1). (C-F). Quantification of the ratio of Pf-5 versus mutant strains at three positions sampled along the radius of the swarming colony: centre, at the inoculation site in the centre of the Petri dish; halfway, at point halfway between the centre and the edge; edge. The Pf-5: mutant ratio was determined based on CFU counts from cell samples taken from the three positions in the co-swarming colony at 36 hrs after inoculation. (C) Ratio of Pf-5 versus the Δ*ofaA* mutant. (D). Ratio of Pf-5 versus the Δ*gacA* mutant. (E) Ratio of Pf-5 versus the Δ*flhA* mutant. Ratio of Pf-5 versus the Δ*gacAflhA* mutant.

The results showed that both mutants 'hitch-hiked' with the wildtype but exhibited distinctly different spatial distribution patterns in the co-swarming colonies (Fig. 1C, 1D). The *ofaA* mutant was located mainly in the centre or within central half of the co-swarming colony but was not detected on the edge of the swarming colony (Fig. 1C). This result is consistent with results described recently for cheater cells of expanding populations of *Saccharomyces cerevisiae* (Datta *et al.*, 2013)2013. In contrast, the *gacA* mutant represented on average 17% of the cells located in the centre of the co-swarming colony, but dominated the bacterial population on the edge of the swarming colony with 89% of the cells (Fig. 1D). These results suggest that in the centre of the swarming colony, the *gacA* mutant, but not the *ofaA* mutant, suffers from competition with the wildtype. These results further revealed that genetically-distinct 'social cheaters', which cannot swarm on their own, exhibit very different spatial distribution patterns during range expansion of a mixed microbial population. The *ofaA* mutant moves behind the wildtype, whereas the *gacA* mutant moves together and ahead of the wildtype during co-swarming.

Flagella are known to be essential for swarming motility (Minamino & Macnab, 1999, Li & Sourjik, 2011). Accordingly, a mutation in *flhA*, which encodes a component of the flagellar export apparatus, eliminated swarming motility of Pf-5 (Fig. 1A) as observed previously (Hassan *et al.*, 2010). When co-inoculated with wildtype Pf-5, the *flhA* mutant was only found in the centre of the colony and did not disperse outwards (Fig. 1B; 1E). When we mutated *flhA* in the *gacA* mutant background and conducted a similar co-swarming experiment, the double mutant *gacA*-Δ*flhA* was detected only in the centre, albeit at low densities, but was not detected at sampling sites halfway along the radius or at the edge of the swarming colony (Fig. 1F). These results indicate that the *gacA* mutant requires an intact flagellar apparatus to co-swarm with the wildtype and to disperse to the edge of the colony.

Successive swarming of wildtype Pf-5 leads to accumulation of *gacA/S* spontaneous mutations on the edge and causes colony collapse

The distinct proliferation of the *gacA* mutant on the edge of the co-swarming colony combined with previous observations that several *Pseudomonas* species are prone to spontaneous mutations in the *gacS/gacA* regulatory system (Bull *et al.*, 2001, Sanchez-Contreras *et al.*, 2002, van den Broek *et al.*, 2005, Driscoll *et al.*, 2011) led us to investigate: i) if spontaneous mutations in the GacS/GacA regulatory system occur during swarming of wildtype Pf-5, ii) if these spontaneous mutants accumulate on the edge of the swarming colony, and iii) if this accumulation compromises colony expansion and dispersal of wildtype Pf-5. To that end, we set-up experimental evolution experiments similar to that of Velicer and Yu (2003)@ and Van Ditmarsch et al (2013). Specifically, cells from the edge of a swarming colony of wildtype strain Pf-5 were transferred successively to new swarm plates (Fig. 2), serving as inoculum for the next swarming colony. During a total of ten

repeated rounds of 'swarming cycles', the diameter of the swarming colony of Pf-5 was measured and cells at the centre and edge of the swarming colony were collected. We then determined the percentage of cells deficient in extracellular protease production, a readily visualized phenotype of *gacS/gacA* mutants of Pf-5 (Fig. 2). Two types of exoprotease mutants were observed during the course of the experiment (Fig. S1).

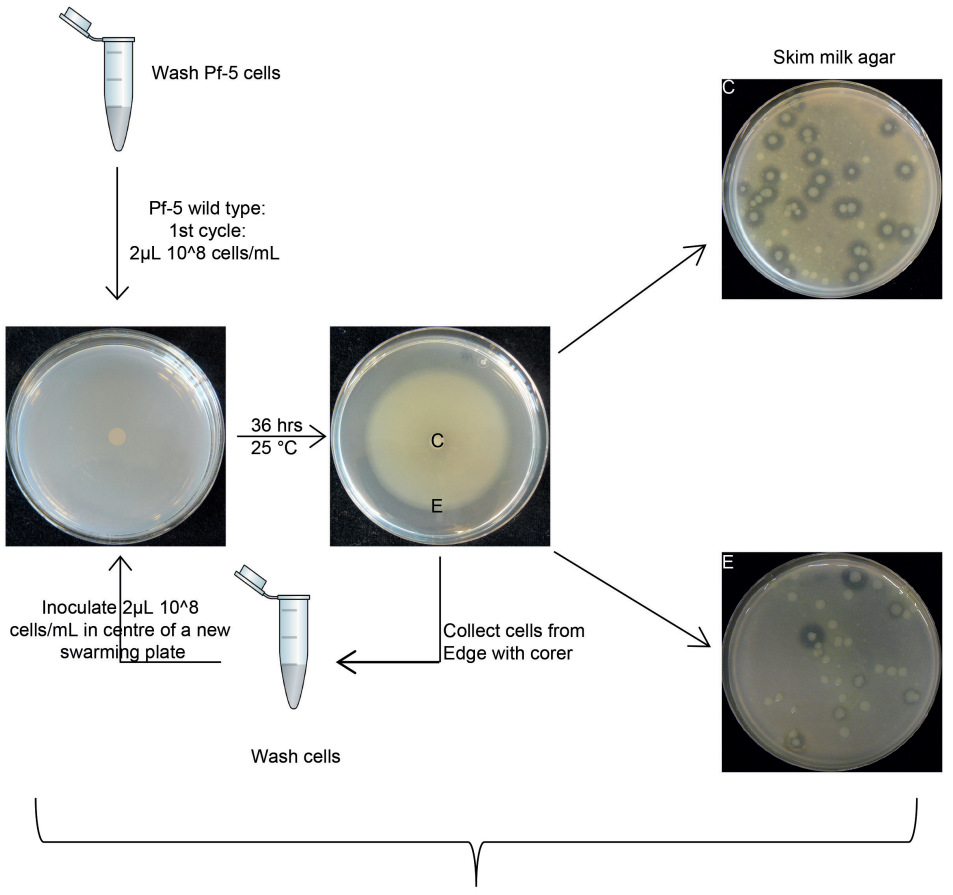

Figure 2. Experimental setup to determine the evolution of spontaneous *gac* mutations in wildtype *P. protegens* Pf-5. Wildtype Pf-5 was inoculated (2µL of $10^{\wedge 8}$ cells/ml) in the centre of soft agar (0.6% w/v) plates and incubated at 25°C for 36 hrs. Cells from the margins of the swarming colony were collected, washed, set to a density of $10^{\wedge 8}$ cells/ml and re-inoculated in the centre of a fresh swarming plate. This process was repeated for 10 successive cycles. For each of the swarming cycles, cells were collected from the centre and edge of the swarming colony after 36 hrs of incubation and dilution plated on skim milk agar (SMA) to determine the frequency of exoprotease-deficient cells, a phenotype typically associated with *gacA/gacS* mutants.

In the first and second cycles, the diameter of the swarming colony was approximately 60 and 45 mm, respectively, after 36h of incubation at 25°C (Fig. 3A, 3B). From the

3rd cycle onwards, the diameter of the swarming colony further decreased and in the 6th cycle the colony collapsed, *i.e.*, there was no or little outward swarming from the inoculation point (Fig. 3A, 3B). This colony collapse coincided with an increase in the frequency of exoprotease-deficient cells on the edge of the swarming colonies (Fig. 3C).

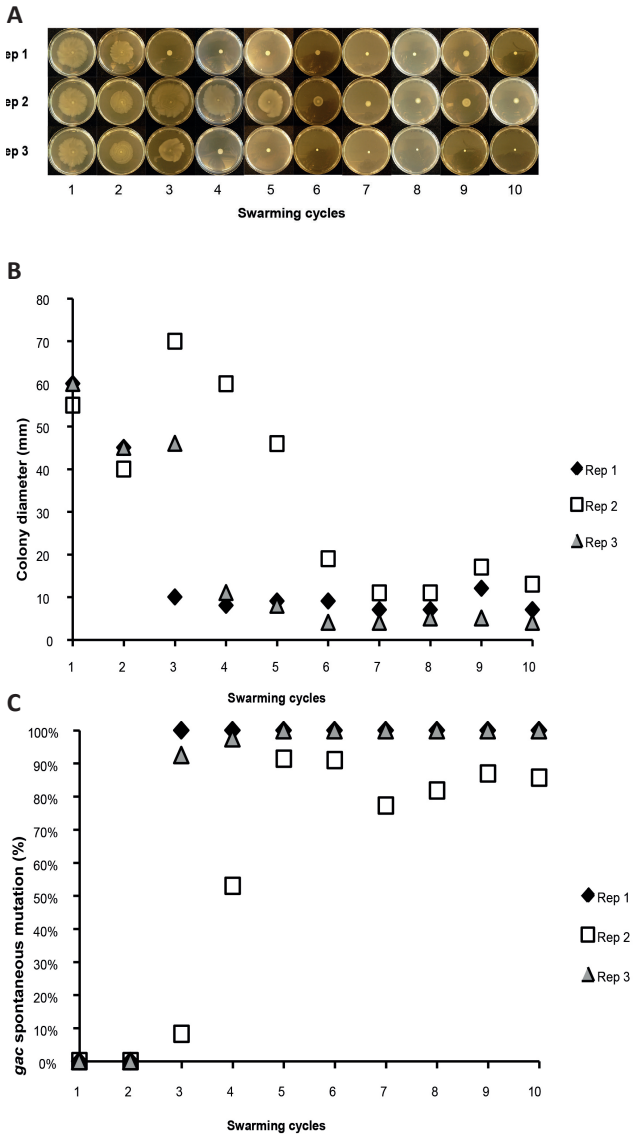

Figure 3. Frequency of *gac* spontaneous mutations during successive swarming of wildtype *P. protegens* Pf-5. (A). Three biological replicates (1-3) were subjected to experimental evolution by successive passages of growth on swarming media. (B). Colony diameter of wildtype strain Pf-5 during 10 successive swarming cycles for three biological replicates separately. (C). Frequency of *gac* spontaneous mutations of wildtype Pf-5 cells collected from the edge of swarming colonies for each of the 10 successive cycles. The *gac* spontaneous mutation rates were calculated in each cycle based on the frequency of exoprotease-deficient cells detected on SMA plates (see Fig. 2; Fig S1).

At the end of the third cycle, on average 67% (± 29% SEM) of the cells from the edge of the swarming colony lacked exoprotease production, whereas on average 33% (± 31% SEM) of the cells in the centre of the colony showed this phenotype (Fig. 3C). In cycles 4 to 10, the frequency of exoprotease-deficient cells on the edge increased to an average of 90% (± 15% SEM) (Fig. 3C). For the three biological replicates, substantial

variation in swarming was observed, but for each of these replicates colony collapse coincided with the accumulation of spontaneous exoprotease-deficient mutants to a frequency of approximately 95% (Fig. 3C). These results suggest that the accumulation of putative *gacS/gacA* mutants on the edge of the colonies during successive swarming contributed, at least in part, to the colony collapse of wildtype Pf-5. To further support this conclusion, we co-inoculated swarming plates with cell suspensions of the *gacA* mutant and wildtype Pf-5 mixed at different ratios and then measured the diameter of the swarming colony. The results from two independent experiments showed that *gacA*:wildtype ratios of at least 3:1 led to a collapse of the swarming colony (Fig. 4).

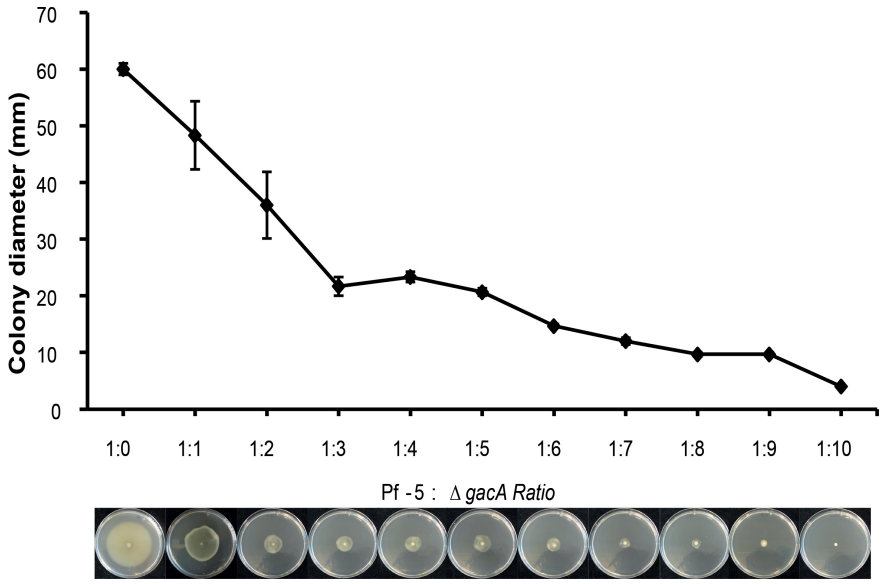

Figure 4. Effect of different cell density ratios of wildtype Pf-5:Δ*gacA* (1:1 to 1:10) on the diameter of the swarming colony. X axis shows the initial ratio Pf-5:Δ*gacA*. Mean values of three biological replicates are given and the error bar represents the standard error of the mean.

We then set out to compare frequencies of *gacS/gacA*-mutant accumulation under swarming vs. non-swarming conditions. For experiments done in non-swarming conditions, cell suspensions of wildtype Pf-5 were spread on 1.5% (w/v) agar plates to obtain a confluent colony with a diameter of approximately 55-60 mm. After incubation for 36h at 25°C, cells from the edge of the colony were used as inoculum for spread plating the next confluent colony. Also here, the frequency of cells with the exoprotease-deficient phenotype was monitored in the centre and edge of the colony for a total of ten successive cycles, as described above. Although the occurrence of spontaneous *gacS/gacA* mutants of Pf-5 during cultivation on nutrient-rich broth media has been reported previously (Whistler *et al.*, 1998), we did not detect spontaneous mutants lacking exoprotease production under the non-swarming conditions and time course used in these experiments (Fig. S2). Collectively, these results suggest that the

occurrence of spontaneous, putative *gacS/gacA* mutations in colonies of wildtype Pf-5 is context dependent.

Genetic characterization of spontaneous mutants that live on the edge

To confirm that the exoprotease-deficient mutants found under swarming conditions indeed have spontaneous mutations in *gacA* or *gacS*, we randomly selected a total of 80 spontaneous exoprotease-deficient mutants of the three biological replicates from swarming cycles 3 thru 10 (Fig. 3C). We then evaluated each mutant for swarming motility and sequenced both the *gacA* (642 bp) and *gacS* (2754 bp) genes. All 80 spontaneous mutants were deficient or reduced in exoprotease activity and had a single point mutation either in *gacA* (N=25), *gacS* (N=51) or yet unknown genes (N=4). For the 25 spontaneous *gacA* mutants, 12 had a point mutation resulting in an E36K substitution in the CheY receiver domain (REC) and 13 had a point mutation resulting in an R205C substitution in the helix-turn-helix domain (Fig. 5A). For the spontaneous *gacS* mutants, six had a point mutation resulting in a D321N substitution in the histidine kinase A domain and two had a point mutation resulting in a G419S substitution in the histidine kinase-like ATPase (HATPase) domain (Fig. 5B). Both domains are known to function in signal transmission of GacS (Heeb & Haas, 2001). All of the other 43 spontaneous *gacS* mutants had a T474M substitution (Fig. 5B). When looking into the frequency and dynamics of each of these *gacA/S* mutations over the successive swarming cycles, the results showed that in cycle 3 all of the detected mutations were T474M substitutions in *gacS* (Fig. 5C). In the subsequent cycles other *gacA/S* mutations emerged, but the T474M mutation in *gacS* prevailed throughout the course of the experiment with a frequency of approximately 40% or higher in swarming cycles 3-10 (Fig. 5C).

For a total of 20 randomly-selected spontaneous mutants (*gacA*, N=13; *gacS,* N=7), we re-introduced the *gacA* or *gacS* genes on plasmids and found that swarming motility and extracellular protease activity were fully restored for 12 spontaneous mutants (Fig. 5D) and partially restored for 8 mutants. Partial complementation may be related to the high copy number of the plasmid-borne *gacA* or *gacS* gene in the bacterial cell, which can disrupt the stoichiometric balance between the sensor kinase and the response regulator that is essential for proper functioning of the system (Cheng *et al.*, 2013). However, we cannot exclude the possibility that (an)other mutation(s) influencing extracellular protease production are present in the eight partially-complemented *gacS/gacA* mutants. Nevertheless, the sequencing and complementation data provide compelling evidence that the vast majority of spontaneous exoprotease-deficient mutants that accumulate on the edge of swarming colonies of Pf-5 have single point mutations in *gacS* or *gacA*.

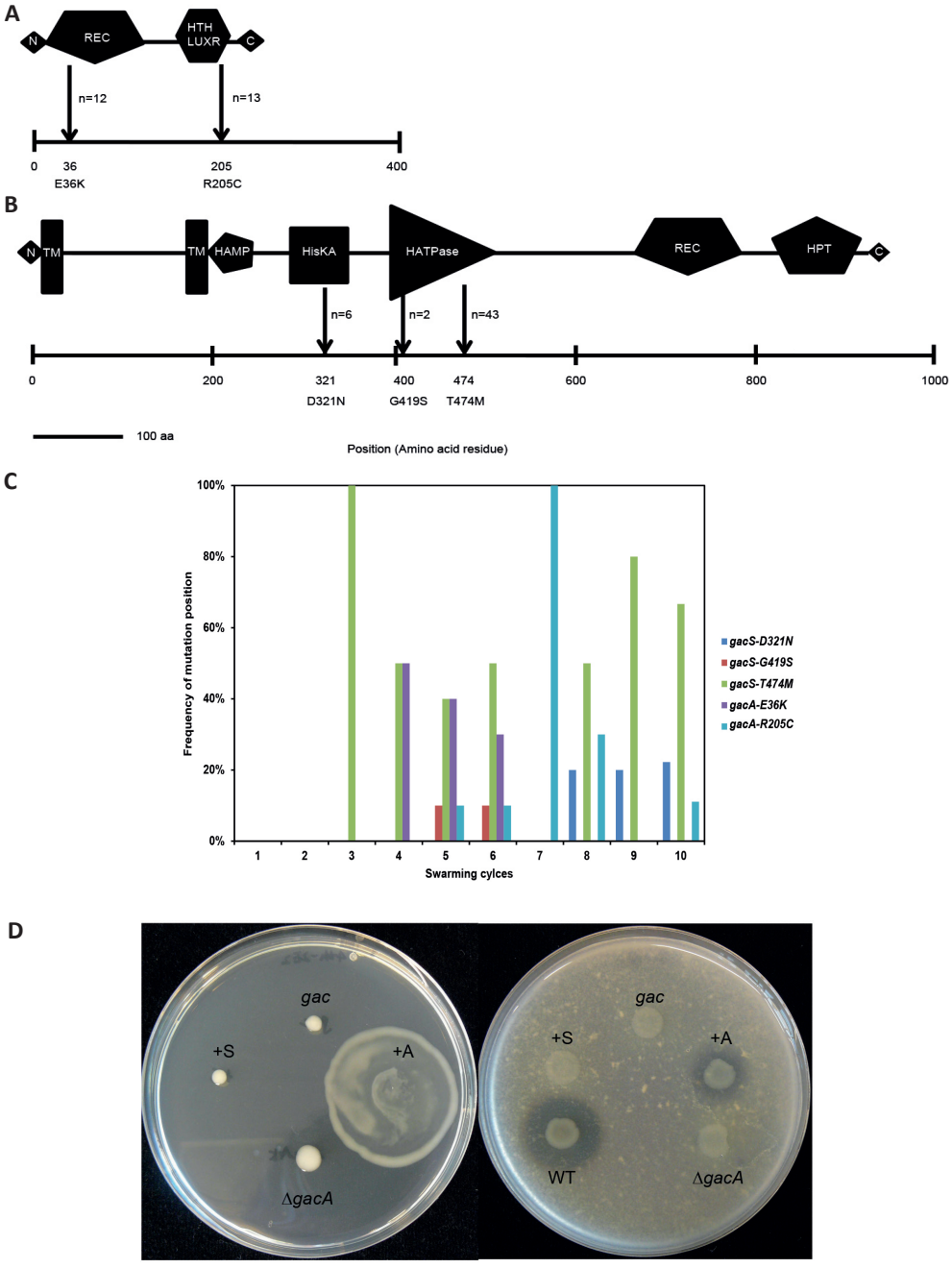

Figure 5. Genetic characterization of the spontaneous mutants that arose during successive swarming of wildtype *P. protegens* Pf-5. (A+B). Schematic representation of the different mutations found in the GacS sensor kinase or the GacA response regulator of Pf-5. Arrows indicate the location of the mutations in GacA (A) or GacS (B) and the amino acid substitutions represent the changes in mutants vs. wildtype allele. Abbreviations: TM: transmembrane segment; HAMP: histidine kinases; HisKA: his kinase A domain (phosphoacceptor); HATPase: histidine kinase-like ATPases; REC: cheY-homologous receiver domain; HPT:

histidine phosphotransfer domain; HTH LUXR: helix_turn_helix Lux regulon. (C). Frequency of mutation position of every swarming cycle. X-axis represents the 10 successive swarming cycles; Y-axis represents the frequency at each mutation position. (D). Swarming motility (left picture) and extracellular protease activity (right picture) of wildtype Pf-5, Δ*gacA* mutant, *gac* spontaneous mutant (*gacA-E36K*) and the *gac* spontaneous mutant (*gacA-E36K*) harboring pME6000-*gacS* (+S) or pME6000-*gacA* (+A), respectively. In the swarming assay (left picture), the wildtype was not included to prevent mixing of colonies. A halo around the colony grown on SMA plates (right picture) is indicative of extracellular protease activity.

Transcriptomics and microscopy provide insight into life on the edge

With their genotypes confirmed, we next asked why *gacS/gacA* mutants accumulate on the margins of swarming colonies of Pf-5. To gain insight into the physiological differences between *gacS/gacA* mutants and wildtype cells under swarming conditions, we characterized the *gac* transcriptome of Pf-5 on swarming medium. A total of 1465 genes were differentially regulated (>2-fold, $P<0.05$) in the *gacA* mutant versus the wildtype, with 705 and 760 genes up- and down regulated, respectively (Table S1). Many of these *gac*-regulated genes confer phenotypes that could influence the distribution or relative fitness of a *gacA* mutant in a swarming colony with wildtype Pf-5. For example, a number of genes involved in motility, chemotaxis, carbon utilization, and ATP synthesis were upregulated in the *gacA* mutant vs. wildtype Pf-5 on swarming medium (Fig. 6). These results suggest that *gac* mutant cells have an increased investment in private goods that could enhance resource acquisition either metabolically or via the exploitation of expanded habitats through motility. This enhanced investment may contribute to the competitive success and relative fitness of the *gacS/gacA* mutants on the edge of swarming colonies.

To further investigate if the observed transcriptional changes in the flagellar genes (Fig. 6) affected the *gacA* mutant phenotype, transmission electron microscopy (TEM) was performed on *gacA, ofaA* and Pf-5 wildtype cells collected from the colony centre from swarming media. The colony centre was chosen since the *gacA* and *ofaA* mutants cannot swarm. The results showed that the *gacA* mutant cells were more flagellated and approximately 1.5 times longer than wildtype Pf-5 and *ofaA* cells (Fig. 7A, 7B).

The *gacA* transcriptome analysis also showed differential expression of many genes for the production of public goods such as orfamide A and other secondary metabolites, exoenzymes and siderophores (Fig. 6). Several of these transcriptional changes in the *gacA* mutant were also observed phenotypically, including the deficiency in orfamide-mediated haemolytic activity, lack of HCN and exoprotease production, and enhanced siderophore production (Fig. 7C). The differential expression of these genes could influence the relative growth of *gacS/gacA* mutants vs. wildtype Pf-5 within a swarming colony. *GacA* mutants of *P. protegens* can grow to higher optical densities than their wildtype (Bull *et al.*, 2001), which is attributed, at least in part, to the released metabolic load associated with the lack of production of secondary metabolites by these mutants.

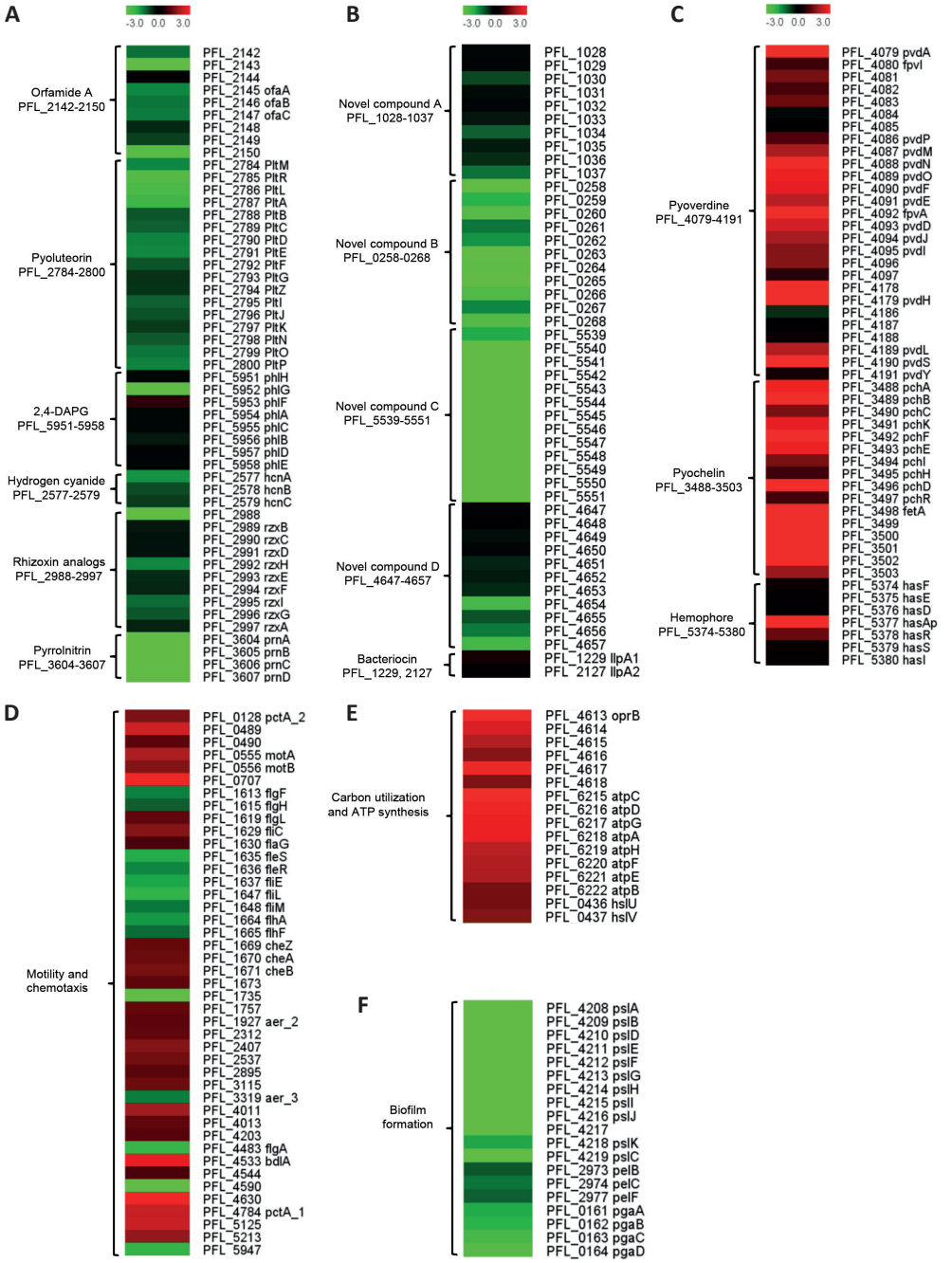

Figure 6. Whole genome transcriptome analysis of *P. protegens* Pf-5 and the *gacA* mutant. Heatmaps showing $\log_2$-fold changes in the expression of genes in the *gacA* mutant vs. wildtype cells in known (A) or putative (B) secondary metabolite biosynthetic gene clusters, (C) siderophore gene clusters, (D) motility and chemotaxis genes, (E) potential carbon utilization and ATP synthesis related genes, (F) biofilm formation. Wildtype Pf-5 and the *gacA* mutant were grown on swarming plates at 25ºC for 36 hrs. Cells of wildtype Pf-5

were collected from the centre of the swarming colony to match the sampling position with that of the non-swarming *gacA* mutant. Total RNA was extracted followed by cDNA synthesis, labelling and hybridization to a Pf-5 whole-genome tiling microarray with 133,488 60-mer probes. The fold changes shown here represent averages of four biological replicates. All genes differentially regulated in the *gacA* mutant vs. wildtype Pf-5 are listed in Supplementary Table S1.

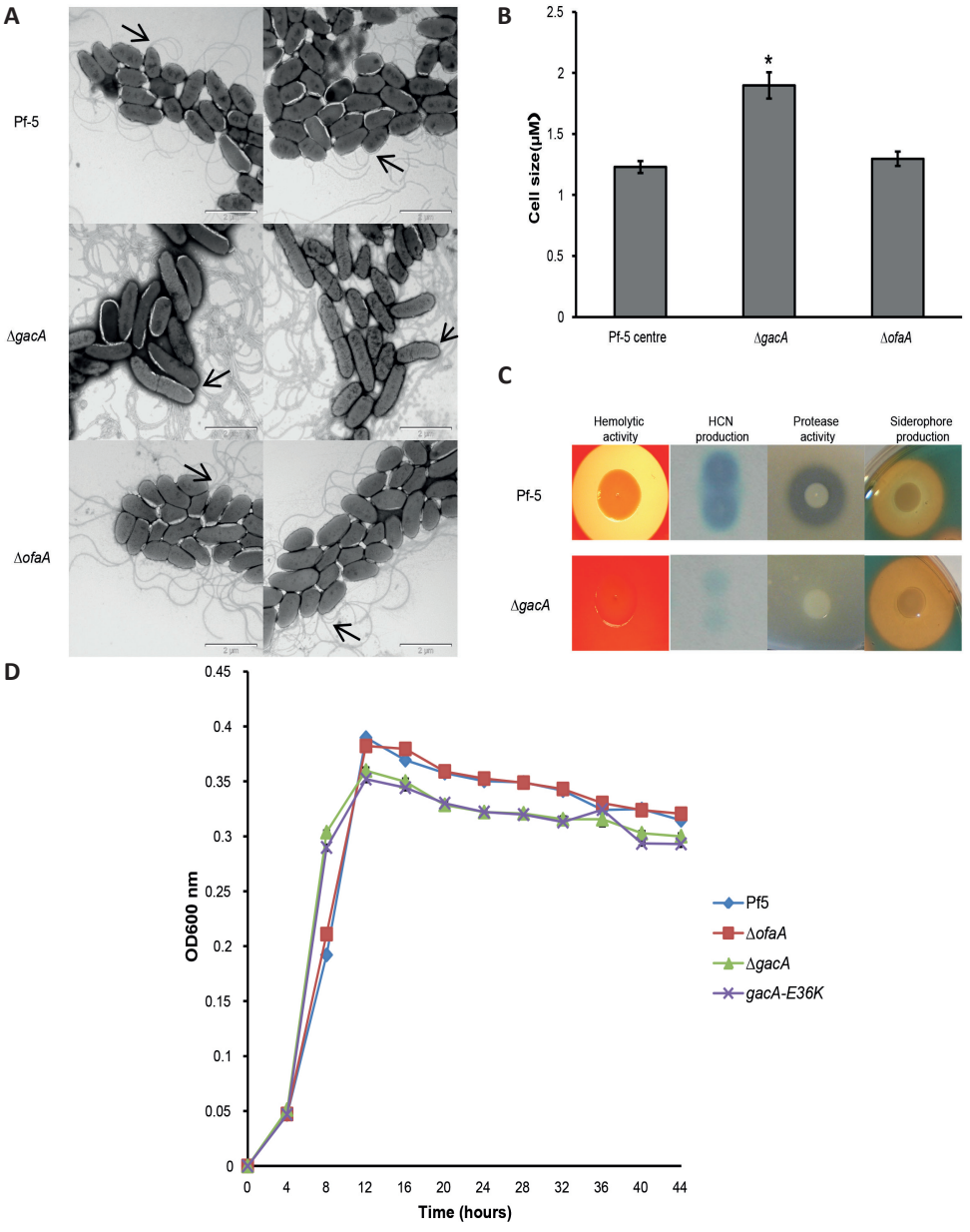

Figure 7. (A). Transmission electron microscope images of *P. protegens* Pf-5, *gacA* mutant and *ofaA* mutant cells negatively stained with 1% phosphotungstic acid (pH 7.2). Bars, 2 µm. The arrows point at polar flagella. Two representative photos are shown for each strain. (B). Cell size of *P. protegens* Pf-5 and mutants as determined by transmission electron microscope. Mean values of 20 randomly selected cells are given

and error bars represent the standard error of the mean. The asterisk indicates a statistically significant (P<0.05) difference from wildtype Pf-5. (C). Phenotypes associated with secondary metabolite production of *P. protegens* Pf-5 and **Δ***gacA* mutant. Hemolytic activity, hydrogen cyanide (HCN) production, extracellular protease activity and siderophore production. (D). Growth of wildtype *P. protegens* Pf-5, *ofaA* mutant, Δ*gacA* mutant, and *gacA* spontaneous mutant (*gacA*-E36K) in modified KB broth at 25°C. At different time points, the optical density of the cell cultures was measured spectrophotometrically (OD_{600} nm). Mean values of four biological replicates are given and the error bars represent the standard error of the mean.

Growth studies conducted here showed that in liquid broth, the *gacA* mutants showed a slightly higher growth rate but only in the early exponential phase (Fig. 7D). While genes for secondary metabolism were downregulated in a *gacA* mutant on swarming medium, genes for production of the siderophores pyoverdine and enantio-pyochelin were significantly upregulated in the *gacA* mutant (Fig. 6C). Siderophores function as public goods promoting the growth of both siderophore producing- and non-producing cells in iron-limited environments (West *et al.*, 2007, Ghoul *et al.*, 2014b), and their overproduction may balance the "cheater" role of *gacA* mutants with respect to orfamide A production. While determining the exact mechanisms driving the interactions between *gacS/gacA* mutants and wildtype Pf-5 in swarming colonies was beyond the scope of this study, the whole-genome transcriptome analysis provided insight into the complex roles of private and public goods that are likely to contribute to cooperation in swarming motility.

Conclusions

Lipopeptide surfactants play an essential role in swarming motility of different bacterial genera (Raaijmakers *et al.*, 2010) allowing the exploration and exploitation of new niches and nutritional resources. Here we showed that the lipopeptide orfamide A of *P. protegens* Pf-5 serves as a "public good" promoting swarming motility of both producing (cooperators) and non-producing cells (cheaters) in a context- and frequency-dependent manner. These results extend those obtained previously for rhamnolipid-deficient mutants of *P. aeruginosa* (Venturi *et al.*, 2010, Xavier *et al.*, 2011, de Vargas Roditi *et al.*, 2013), although the orfamides produced by Pf-5, unlike rhamnolipids of *P. aeruginosa*, are not quorum-sensing (QS) regulated public goods. In Pf-5, a mutation in either the orfamide biosynthesis gene *ofaA* or the global regulatory gene *gacA* resulted in loss of orfamide production and swarming motility, but both types of mutants can swarm in the presence of the wildtype. Despite their shared characteristics as 'social cheaters', we observed a striking difference in the spatial distribution of the *gacA* and *ofaA* mutants when co-swarming with wildtype Pf-5, with the *gacA* mutant predominating on the edge of the colony. These results show that different 'social cheaters' exhibit different spatial distribution patterns in swarming colonies. Our experimental evolution experiments further revealed that successive swarming of wildtype *P. protegens* leads to the emergence and accumulation of spontaneous *gac* mutants on the edge, ultimately leading to a collapse in colony expansion. Studies by van Ditmarsch et al.

(2013) showed that successive swarming cycles with *P. aeruginosa* led to the evolution of hyperswarmers, all of which had a point mutation in the flagellar synthesis regulator FleN. The multiflagellated hyperswarmers outcompeted the ancestral strain in swarming competitions and this advantage was growth-rate independent. In this study, we did not detect hyperswarmers of *P. protegens* but instead observed a colony collapse due to the accumulation of cells with a non-swarming phenotype. Despite the strong difference in swarming phenotypes observed, i.e. colony expansion (van Ditmarsch *et al.*, 2013)2013 vs. colony collapse (this study), the similarities between the two studies are striking. From a conceptual perspective, our study showed that surface migration drives parallel evolution toward accumulation of specific spontaneous mutations. Hence, adaptive convergent evolution through point mutations may be a common feature of range-expanding microbial populations. The genotypes of mutants that accumulated on the edge of swarming colonies of *P. aeruginosa* (*fleN*) and *P. protegens* (*gacS/gacA*) differ considerably, but the phenotype of hyperflagellation was common to the mutants of both studies. In *P. aeruginosa*, the advantage of the hyperswarmers was reported to be growth-rate independent, whereas a *gacA* mutant of *P. protegens* has a slightly higher growth rate than the wildtype in the early exponential phase. Our whole-genome transcriptome analyses confirmed that several genes involved in motility and chemotaxis were upregulated in *gacA* mutants under swarming conditions, and also revealed that various genes associated with biofilm formation were down-regulated (Fig. 6F). This further extends the mounting evidence for an inverse regulation and evolutionary trade-off between motility and biofilm formation in bacteria. It should be emphasized that in the experimental evolution assays we limited our screening to cells with spontaneous mutations influencing extracellular protease production, the vast majority of which were in the *gacA* or *gacS* genes. Whether other spontaneous mutations accumulated on the edge or elsewhere in the swarming colony remains to be tested.

In the ecological context of the rhizosphere, where plant-associated bacteria encounter numerous competitors, predators and phages, *gacS/gacA* mutants could function as scouts in the colonization of new habitats. Similar to the hyperswarmers of *P. aeruginosa*, *gacA* mutants of *P. protegens* Pf-5 are poor biofilm formers (Kidarsa *et al.*, 2013) and likely face strong counterselection in soil and rhizosphere environments where biofilms are assumed to provide protection against competitors and protozoan predators. Work by Friman and Buckling (2013) on interactions between *P. fluorescens*, a virus and a predatory protist elegantly highlighted the complexity of these co-evolutionary dynamics in structuring natural communities and maintaining diversity. With respect to the behavior of *gac* mutants in soil and plant-associated environments, work by Chancey et al. (2002) with *P. chlororaphis* strain 30-84 demonstrated that although the mutant population partially displaced the wildtype in sterile soil, it did not do so in natural soil. Work on *P. fluorescens* F113 by Sanchez-Contreras et al. (2002) and Martinez-Granero et al. (2006), however, suggested that during rhizosphere colonization *gac* mutants

were found among the phenotypic variants and these exhibited an enhanced motility. Whether *gacS/gacA* mutants on the edge of a swarming colony in the rhizosphere could serve as a 'domesticated' food source for amoebae and protozoa, as shown recently by Stallforth et al. (2013), or as scouts to benefit the rest of the swarming colony remains to be addressed.

Materials and Methods

Strains, Growth Conditions and swarming assay. Bacterial strains used in this study are *P. protegens* strain Pf-5 (Howell & Stipanovic, 1979) and its mutants Δ*ofaA* (Gross *et al.*, 2007b)2007b, Δ*gacA* (Hassan *et al.*, 2010), and Δ*flhA* (Hassan *et al.*, 2010). Strains were cultured in King's medium B (KB) (20 g/L oxoid proteose peptone, 1.5 g/L $MgSO_4$*7 H_2O, 1.2 g/L KH_2PO_4, 10 g/L glycerol). KB plates were solidified with 1.5% (w/v) oxoid technical agar. Antibiotics were added at the following final concentrations: streptomycin 100 µg/ml, kanamycin 100 µg/ml, or tetracycline 200 µg/ml, respectively. Swarming assays were conducted largely according to the method described by de Bruijn and Raaijmakers (de Bruijn & Raaijmakers, 2009), except the medium used here was modified KB soft agar: 10 g/L oxoid proteose peptone, 1.5 g/L $MgSO_4$*7 H_2O, 1.2 g/L KH_2PO_4 with 0.6% (w/v) oxoid technical agar and the inoculum of 2µl of $10^{\wedge 8}$ cells/ml. Bacterial suspensions were prepared from single strains or combinations in 1:1 ratio for (co)swarming assays. Plates were incubated at 25°C for 36-48 hours.

Frequency of *gac* spontaneous mutation under swarming conditions. Swarming assays were conducted as described above. After 36 hours of incubation at 25°C, the samples were taken from the centre and edge of the swarming colony with a 5.0-mm-diameter corer (Fig. 2). The samples were then resuspended in sterile distilled water, diluted and spread plated on Skim Milk Agar plates (10 g/L skim milk powder, 4 g/L oxoid blood agar base, 0.5 g/L yeast extract, 13.5 g/L oxoid technical agar). Cells sampled at the edge of the swarming colonies were washed, set to a fixed density and inoculated to a new swarming plate; this was repeated for a total of 10 cycles (Fig. 2).

Sequencing and complementation of the *gac* spontaneous mutants. Plasmids pME6000-*gacS* (pJEL5999) and pME6000-*gacA* (pJEL5965) contain, respectively, the *gacS* and *gacA* genes from Pf-5 cloned into pME6000. The cloning vector pME6000, which contains the origin of replication from pBBR1 and confers tetracycline resistance, was a gift from Stephen Heeb and Dieter Haas (University of Lausanne). pME6000-*gacS* and pME6000-*gacA* were electroporated into the spontaneous *gacA* and *gacS* mutants. Transformed cells were plated on KB supplemented with tetracycline (25 µg/mL) and the presence of pME6000-*gacS* or pME6000-*gacA* was verified by PCR analysis with primers specific for the tetracycline resistance gene.

Transcriptome analyses

Wildtype Pf-5 and the *gacA* mutant were grown on swarming plates at 25ºC for 36 hrs. Cells of the wildtype were collected from the centre of the swarming colony to match the position with that of the non-swarming *gacA* mutant. Total RNA was extracted with Trizol reagent (Invitrogen) and further purified with the NucleoSpin RNA kit. A tiling microarray for *P. protegens* Pf-5 was developed in the MicroArray Department (MAD), University of Amsterdam (UvA), Amsterdam, the Netherlands. In total, 133,488 probes (60-mer) were designed with, in general, a gap of 46 nucleotides between adjacent probes on the same strand and an overlap by 7 nucleotides when regarding both strands. In addition, 5,000 custom negative control probes were hybridized, and used as an internal control to validate the designed probes in a CGH experiment of 4 arrays. Probes were annotated and assembled into probe sets for known genes based on location information retrieved from the Pathosystems Resource Integration Center (PATRIC, http://patricbrc.org). Probes outside of known genes were labelled as InterGenic Region (IGR). cDNA labelling was conducted as described previously (de Knegt *et al.*, 2013). Briefly, cDNA was synthesized in presence of Cy3-dUTP (Cy3) for the test samples and with Cy5-dUTP (Cy5) for the common reference. The common reference was made by an equimolar pool of the test samples (3 µg per sample). 5 µg of total RNA per reaction was used and yielded 1.5-2.5 µg cDNA for each sample with larger than 16 pmol of Cy3 or Cy5 dye per microgram. Hybridizations were performed according to Pennings et al. (2011). Slides were washed according to the procedures described in the Nimblegen Arrays User's Guide - Gene Expression Arrays Version 5.0 and scanned in an ozone-free room with a Agilent DNA microarray scanner G2565CA (Agilent Technologies). Feature extraction was performed with NimbleScan v2.5 (Roche Nimblegen). Data pre-processing consisted of $\log_2$-transformation of the raw probe-intensity data, followed by a within slide lowess normalization. Thus normalized sample (Cy3) channel intensities were summarized into probe sets values and normalized between arrays using the RMA (Robust Multi-Array Analysis) algorithm (Irizarry, et al. 2003). All results described were found to be significant using a false discovery rate of less than 5%. Analysis of the gene expression data was conducted by Arraystar software.

Transmission Electron microscopy

Swarming assays of bacterial strains Pf-5, *gacA* and *ofaA* mutants were conducted as described above. After 36 hours of incubation at 25°C, the cells were taken from centre of the swarming colony with a sterile inoculation loop. The cells were then resuspended in 100 µL 10 mM ammonium acetate (pH 7.2). Five microliters of the bacterial suspension were placed on 200-mesh carbon coated grids. The excess liquid was removed using a filter paper after 2 min. The bacteria were then negatively stained using 1% phosphotungstic acid (pH 7.2) for 30 seconds. Transmission electron micrographs were obtained using a JEM 1011 (JEOL Ltd., Tokyo, Japan) electron microscope operating on an accelerating voltage of 80 kV.

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Conflict of Interest Statement

The authors of this manuscript have no conflicts of interest to declare.

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Supplementary data

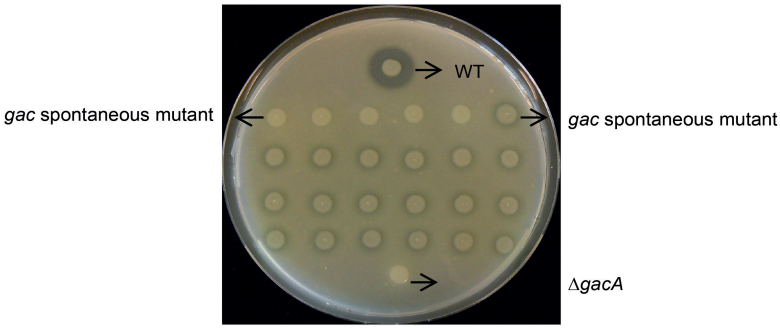

Figure S1. Extracellular protease production of *gac* spontaneous mutants on skim milk agar (SMA). Wildtype Pf-5 and *gacA* mutant serve as positive and negative controls. *Gac* spontaneous mutants showed either no or a slight extracellular protease activity.

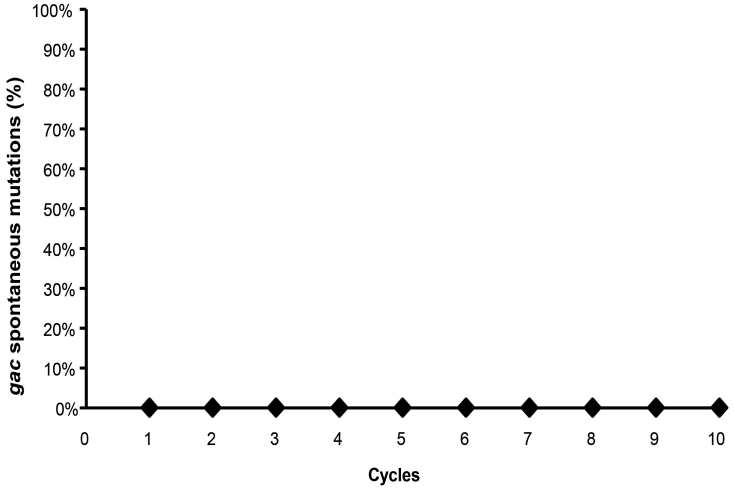

Figure S2. Evolution of spontaneous *gac* mutations in wildtype *Pseudomonas protegens* Pf-5 under non-swarming conditions. Wildtype Pf-5 (2µL of 10^8 cells/ml) was spread plated on solid agar plates (1.5% w/v) to a colony diameter similar to that obtained under swarming conditions (0.6% w/v). After incubation at 25°C for 36 hrs, cells from the margins of the colony were collected, washed, set to a density of 10^8 cells/ml and re-spread to the similar colony size on a fresh solid plate. This process was repeated for 10 successive cycles. For each of the cycles, cells were collected from the edge of the swarming colony after 36 hrs of incubation and dilution plated on skim milk agar (SMA) to determine the frequency of exoprotease-deficient cells, a phenotype typically associated with *gacA/gacS* mutants.

Due to the large size, supplementary table 1 is not shown here, but it is available upon request.

Chapter 7

Summarizing Discussion and Conclusions

Over the past years, substantial progress has been made worldwide in the genome-based discovery of new biosynthesis genes of lipopeptides (LP), surface-active antimicrobial compounds produced by *Pseudomonas* and other bacterial genera. In contrast, relatively little progress has been made on the identification of regulatory genes and signal transduction pathways affecting LP biosynthesis. Therefore, the aims of my PhD thesis were i) to identify new regulatory genes of LP biosynthesis in *Pseudomonas fluorescens*, and ii) to unravel natural functions of LPs in *Pseudomonas*. The work presented led to the identification of four novel regulatory genes (*prtR*, *phgdh*, *dnaK*, *clpA*) and two small RNAs (RsmY, RsmZ) involved in massetolide biosynthesis in *P. fluorescens* SS101 (**chapters 2, 3 and 4**). Meanwhile, we also identified predation-specific responses at the interface of protozoa-*Pseudomonas* interactions by using a unique combination of whole-genome transcriptome analysis, MALDI-TOF-based imaging mass spectrometry (IMS) and live colony NanoDESI mass spectrometry. We showed, for the first time, site-specific and real-time production of the LPs massetolide and viscosin at the interface of *Pseudomonas*-protozoa interaction. The closely related *P. fluorescens* strains SS101 and SBW25 exhibited common transcriptomic and metabolic responses to protozoan predation, but also displayed unique responses (**chapter 5**). In **chapter 6**, the role of LPs in swarming motility of *Pseudomonas* was studied. Experimental evolution assays with repeated swarming cycles of *P. protegens* Pf-5 led to accumulation of spontaneous *gacS/gacA* mutants on the edge of the swarming colony, ultimately causing colony collapse. Potential functions of the accumulation of these spontaneous mutants living on the edge of swarming colonies were explored by phenotypic and transcriptomic analyses.

Regulation of lipopeptide biosynthesis

In *P. fluorescens,* the two-component regulatory system GacS/GacA acts as a master switch: a mutation in either one of the two genes shuts down the production LPs and several other secondary metabolites (Dubern *et al.*, 2005, Kitten *et al.*, 1998, Koch *et al.*, 2002, de Bruijn & Raaijmakers, 2009, Vallet-Gely *et al.*, 2010). To unravel the downstream elements of the GacS/GacA regulatory pathway in *P. fluorescens* SS101, a genome-wide search for small RNAs (sRNAs) was conducted and combined with whole-genome transcriptomic analyses to identify genes associated with the Rsm (repressor of secondary metabolites) regulon. *In silico* analysis led to the identification of RsmY and RsmZ in the SS101 genome, and transcriptomic profiling showed that both sRNAs genes are under the control of GacS/GacA (Chapter 2). In frame deletion of these two sRNAs showed that the Rsm system regulates massetolide biosynthesis via the two repressor proteins RsmA and RsmE, with the LuxR-type transcriptional regulator MassAR as their most likely target. Transcriptome analyses of the *rsmYZ* double mutant further revealed that genes associated with iron acquisition, motility and chemotaxis were significantly upregulated, whereas genes of the type VI secretion system were down regulated. Comparative transcriptomic analyses suggested that most, but not all the genes controlled by RsmY/RsmZ are also controlled by the GacS/GacA two-component

system. Collectively, these results demonstrated that the Rsm regulon plays a critical role in the regulation of LPs and other traits associated with antimicrobial activity. Further experiments are needed to confirm that *massAR* is indeed the target of the repressor proteins. Although the GacS/GacA two-component system has been studied intensively, the signal(s) that serve as a trigger still remain elusive. Koch et al (2002) observed that exudates of sugar beet seeds contain triggers for amphisin production in *Pseudomonas* DSS73. The triggering compound(s) was (were) heat stable and could be removed by dialysis. Koch et al (2002) suggested that the triggers could be small organic compounds but the identity of these molecules is yet unknown. Identifying signal(s) that activate the Gacs/GacA system remains a challenge also considering the relatively poor conservation of the periplasmic loop domains of the GacS sensor kinase among bacterial strains, species and genera. As suggested initially by Heeb and Haas (2001) , it is more likely that the periplasmic loop domain possesses modulatory functions in response to different environmental signals.

To further understand how LP biosynthesis is regulated in *P. fluorescens* SS101, we screened approximately 8,000 random plasposon mutants for reduced or loss of LP production with the aim to identify new regulatory genes (Chapter 3). Out of a total of 58 putative mutants, 45 had a mutation in one of the three massetolide biosynthesis genes *massA*, *massB* or *massC*. For 5 mutants, the insertions were located in the known regulatory genes *gacS, gacA,* and *clpP*. The insertions of the remaining 8 mutants were located in *phgdh,* a gene encoding D-3-phosphoglycerate dehydrogenase, in the heat shock protein encoding gene *dnaK*, in a gene *prtR* encoding transmembrane regulatory protein*,* or in the ClpP chaperone gene *clpA*. Genetic, chemical and phenotypic analyses showed that *phgdh*, *dnaK* and *prtR* are indeed involved in the regulation of massetolide biosynthesis, most likely by transcriptional repression of the LuxR-type regulatory genes *massAR* and *massBCR*. In addition to their role in massetolide biosynthesis, *dnaK* and *prtR* were also found to affect siderophore and extracellular protease(s) production, respectively. In previous studies, it was shown that addition of proline and glutamic acid to the growth medium can partially complement the deficiency of swarming motility in the *clpP* mutant (de Bruijn & Raaijmakers, 2009). In Chapter 3, we showed that *phgdh* is a key gene in L-serine biosynthesis, supporting and extending our initial hypothesis that amino acid metabolism, and especially serine biosynthesis, affects massetolide production.

In chapter 4, evidence is presented that the chaperone ClpA, together with the serine protease ClpP, regulates massetolide biosynthesis in *P. fluorescens* SS101. Whole-genome transcriptome analyses of *clpA* and *clpP* mutants showed their involvement in the transcription of the massetolide biosynthesis genes *massABC* and of the pathway-specific transcriptional regulator *massAR*. Moreover, transcription of genes associated with cell wall and membrane biogenesis, energy production and conversion, amino acid transport and metabolism, and pilus assembly were altered by mutations in *clpA* and *clpP*. Proteome analysis provided insights into putative additional cellular changes associated with *clpA* and *clpP* mutations. In particular, the productions of proteins of

the citrate cycle and the heat shock proteins DnaK and DnaJ were affected in these mutants. Combined with previous findings, these results suggest that the ClpAP complex regulates massetolide biosynthesis via the pathway-specific, LuxR-type regulator MassAR, the heat shock proteins DnaK and DnaJ, and possibly proteins of the TCA cycle. It should be noted that ClpAP is a degradative protease system thereby complicating the interpretation of proteomics data. Hence, the higher abundance of a particular protein in the *clpA* and/or *clpP* mutants can also be due to an inherent up- or down-regulation by other modulated pathways. Results of whole-genome microarray analyses of the *gacA* mutant of strain SS101 (Chapter 2), suggested that expression of *phgdh, dnaK*, *prtR* or *clpA* is not under the control of the GacS/GacA system. Hence, in the regulation model shown in Figure 1, the Gac-signal transduction route is kept separate from the other regulatory genes. The identification of new regulatory genes substantially extended insights into the regulatory network of LP biosynthesis in *P. fluorescens* and into the regulation of other traits that may contribute to its life-style in the rhizosphere and phyllosphere. Disentangling the connection, if any, between the ClpP/ClpA degradation machinery and the GacS/GacA signal transduction pathway will be worthwhile to pursue.

Natural functions of lipopeptides

Pseudomonas fluorescens produces LPs with broad-spectrum antimicrobial activities. Recent studies suggested that these LPs are involved in defense against the protozoan grazer *Naegleria americana*, both *in vitro* and in rhizosphere environments (Mazzola *et al.*, 2009). Genome-wide transcriptome analyses of *P. fluorescens* strain SS101 revealed that upon protozoan grazing, 55 genes were up-regulated and 73 genes were down-regulated (Chapter 5). The LP biosynthesis genes *massABC,* as well as genes involved in alkane degradation and in putrescine catalysis were significantly up-regulated in the *Pseudomonas*-protozoa interaction. Subsequent bioassays revealed that putrescine induced encystment of *N. americana* trophozoite and adversely affected cyst viability. MALDI imaging mass spectrometry (IMS) and live colony NanoDesi mass spectrometry further showed, for the first time, site-specific and real time LP production at the interface of *Pseudomonas*-protozoa interactions (Figure 1). Similar overall transcriptional and metabolic responses were observed when *P. fluorescens* strain SBW25 was exposed to *N. americana*, next to also strain-specific responses. Collectively these results indicate that closely related *Pseudomonas* strains exhibit common transcriptomic and metabolic responses to protozoan predation and display unique responses. The identities and possible functions of the yet unknown metabolites observed during *Pseudomonas*-protozoa interactions will be addressed in future experiments. In *P. aeruginosa*, it was reported that ExoU and other T3SS effectors are required for colonization and killing of protozoa (Matz *et al.*, 2008). Strains of the *P. fluorescens* group also possess T3SS and putative effector proteins but their functions in anti-predation are as yet unknown. Moreover, the potential synergistic effects of LPs, putrescine and the other yet unknown

metabolites in defense against protozoan grazing will be worthwhile to look into. Another intriguing question is which protozoan signals induce LP biosynthesis genes. This may lead to the identification of the unknown triggers of the GacS/GacA two-component regulatory system discussed above. Whole genome sequencing and transcriptomic analyses of the protozoa combined with chemical analysis of its lysate might shed light on the identity of the signals that trigger LP biosynthesis in *Pseudomonas*.

Swarming motility is a flagella-driven multicellular behavior that allows bacteria to colonize new niches and escape competition. In Chapter 6, we investigated the role of LPs in swarming motility of the rhizosphere bacterium *Pseudomonas protegens* Pf-5 and determined the spatial distribution and evolution of 'social cheaters' in swarming colonies. We showed that the LP orfamide and the flagellar machinery of Pf-5 are essential for swarming motility. Orfamide-deficient mutants, with deletions in the biosynthesis gene *ofaA* or in the regulatory gene *gacA*, cannot swarm on their own but 'hitch-hiked' with wild type Pf-5. However, distinctly different spatial distributions in co-swarming colonies were observed between the two mutants, with the *ofaA* mutant moving behind the wild type and the *gacA* mutant predominating on the edge. Subsequent experimental evolution assays showed that repeated swarming cycles of wild type Pf-5 drives parallel evolution toward fixation of spontaneous *gacS/gacA* mutants on the edge, ultimately causing colony collapse. Transcriptome analyses of these spontaneous regulatory mutants revealed that genes associated with resource acquisition, motility, chemotaxis and efflux were significantly upregulated. Moreover, *gacA* mutant cells were longer, more flagellated and more tolerant to several antibiotics than wild type cells. These mutations and the concomitant proliferation of social cheaters on the edge can cause colony collapse but, considering segmentation patterns in swarming colonies, may also confer local fitness benefits to the colony when confronted with competitors during dispersal into new and hostile territories. Metabolomics analysis of the (co)swarming colonies and correlation with the whole-genome transcriptomics data might provide more insight into the potential functions of the spontaneous *gac* mutants living on the edge. In the work presented, we only focused on the extracellular protease deficiency phenotypes and therefore limited our study to spontaneous *gac* mutations. Whole genome sequencing of many more cells living on the edge of swarming colonies may unravel other mutations that may also confer specific functions during the dispersal of bacterial colonies. The most intriguing challenge in this context is to measure and visualize swarming motility and microbial interactions in the rhizosphere.

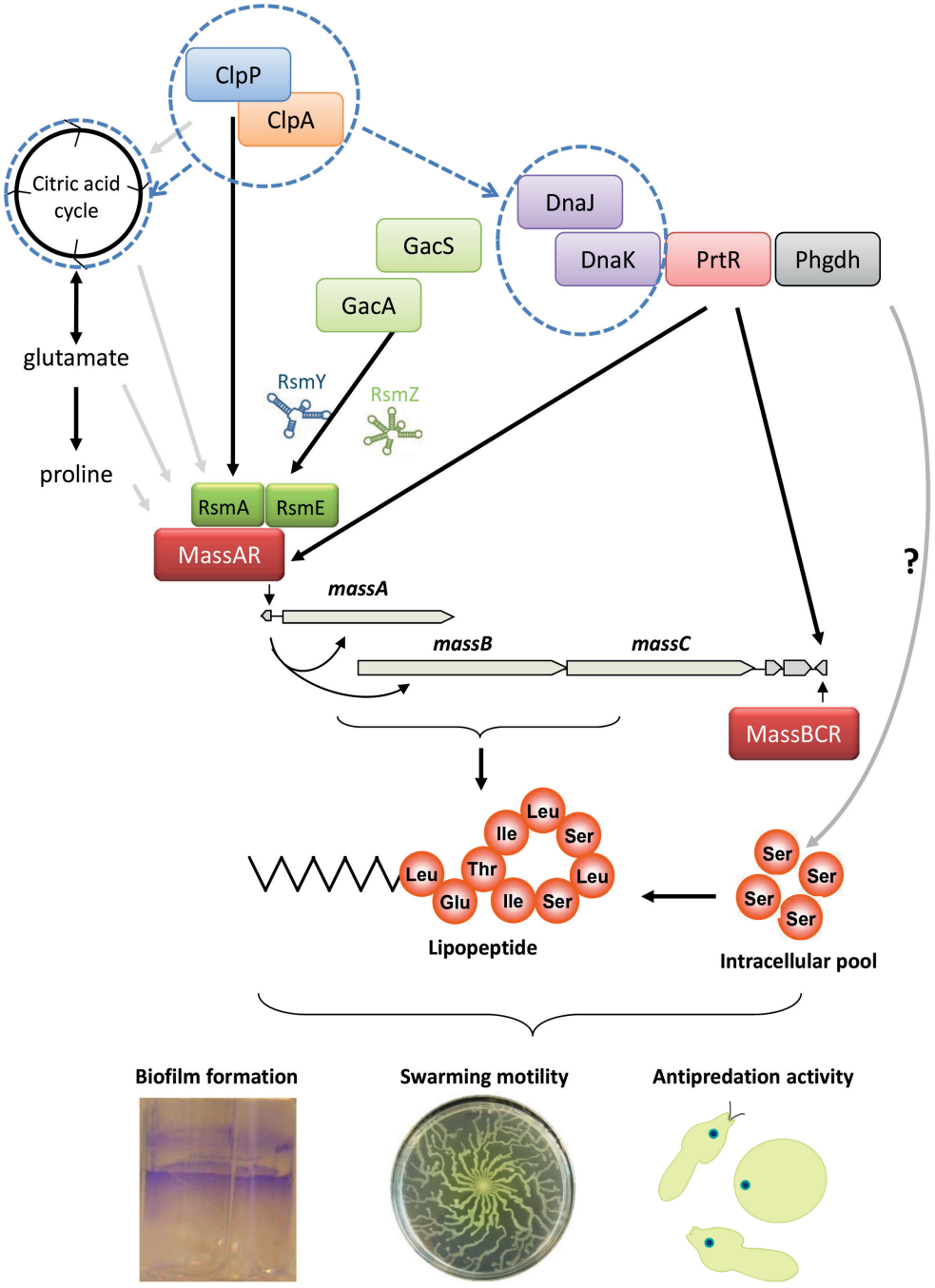

Figure 1. Model for the genetic regulation of LP biosynthesis and natural functions of LP in *Pseudomonas*. The black shaded squares are based on experimental data obtained before; the color shaded squares are based on this thesis.

Future perspectives

In the genome of *Pseudomonas fluorescens* SS101, 350 unique genes were found, most of them with unknown functions or classified as (conserved) hypothetical proteins (Loper *et al.*, 2012). Each of the ten Pseudomonas strains included in the comparative genomic analysis has a reservoir of unique strain specific genes ranging in number from 313 to 930. Understanding the functions of these yet unknown genes as well as the regulatory pathways in bacteria will not only help to unravel the molecular basis of intricate communications between microbes, it also can be exploited to identify and activate cryptic gene clusters of secondary metabolites. The latter will bring new insights and opportunities for natural product discovery, especially given the increasing demand for novel antimicrobial pharmaceuticals to combat multidrug resistant (MDR) pathogens. Manipulating regulatory genes to elicit the production of new natural products from silent biosynthetic gene clusters has been reported (Laureti *et al.*, 2011, Rigali *et al.*, 2008). In *Streptomyces ambofaciens* ATCC23877, constitutive expression of a LuxR-type transcriptional regulator triggered the biosynthesis of a giant type I modular polyketide synthase (PKS) gene cluster and led to the identification of the glycosylated macrolides stambomycins A-D (Laureti et al., 2011). On the other hand, LuxR-type transcriptional regulators can also function as repressors (Yamanaka *et al.*, 2014). Eliminating these repressors combined with cloning and expression of a 67-kb NRPS gene cluster from the marine actinomycete *Saccharomonospora* sp. CNQ-490, led to production of the chlorinated lipopeptide antibiotic taromycin A (Yamanaka et al., 2014). LuxR-type transcriptional regulators are numerous in *Pseudomonas* genomes. Hence, the constitutive expression or suppression of such pathway-specific activators/repressors might also represent a powerful approach for natural product discovery.

In spite of the enormous potential of LPs for therapeutic and environmental applications (Desai & Banat, 1997, Cameotra & Makkar, 2004, Singh & Cameotra, 2004, Rodrigues *et al.*, 2006, Pirri *et al.*, 2009), industrial level production has not yet been realized for many LPs due to their low yields. Therefore, understanding the genetic regulatory mechanisms of LP biosynthesis will help to develop metabolically engineered hyper-producing strains with better product characteristics and acquired capability of utilizing cheap agro-industrial waste products as substrates. Moreover, insight into how the bacteria secrete LPs would also facilitate the production efficiency of these multifunctional metabolites for both environmental and industrial applications.

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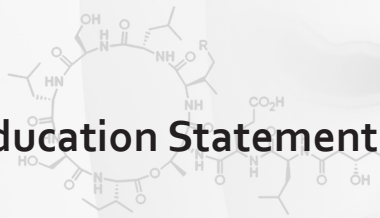

Summary

Lipopeptides (LPs) are surface-active, antimicrobial compounds composed of a lipid moiety linked to a short linear or cyclic oligopeptide. In bacteria, LPs are synthesized by large nonribosomal peptide synthetases (NRPSs) via a thiotemplate process. Compared to the understanding of LP biosynthesis, little is known about the genetic regulation. **The aims of this PhD thesis** were to identify new regulatory genes of LP biosynthesis and to unravel the natural functions of LPs in plant-associated *Pseudomonas* species. Using a combination of various 'omics'-based technologies, we identified two small RNAs, designated RsmY and RsmZ, that, together with the repressor proteins RsmA and RsmE, regulate the biosynthesis of the LP massetolide in the rhizosphere bacterium *Pseudomonas fluorescens* SS101. Four other regulatory genes (*phgdh*, *dnaK*, *prtR* and *clpA*) of massetolide biosynthesis were identified via random mutagenesis. Mutations in each of these four genes caused a deficiency in massetolide production, swarming motility and biofilm formation, two natural functions associated with the production of LPs in *Pseudomonas*. Results further indicated that the ClpAP protease complex regulates massetolide biosynthesis via the pathway-specific, LuxR-type regulator MassAR, the heat shock proteins DnaK and DnaJ, and proteins of the TCA cycle.

LPs exhibit broad-spectrum antimicrobial activities and have diverse natural functions for the producing bacteria. LPs of *P. fluorescens* were shown to play an important role in defense against protozoan predation. Genome-wide transcriptome analysis revealed that 55 and 73 genes were up- and down-regulated respectively in *P. fluorescens* strain SS101 upon grazing by the protozoan predator *Naeglaria americana*. The up-regulated genes included the LP biosynthesis genes *massABC,* but also genes involved in alkane degradation and in putrescine catalysis. Putrescine induced encystment of the protozoa, possibly providing a second line of defense against predation. MALDI imaging mass spectrometry (IMS) and live colony NanoDesi mass spectrometry further revealed, in real time, site-specific LP production at the interface of *Pseudomonas*-protozoa interactions. When the closely related strain *P. fluorescens* SBW25 was exposed to *N. americana*, similar overall transcriptional and metabolic responses were observed as found for strain SS101, but also strain-specific responses were apparent. These results indicate that closely related bacterial strains exhibit common and unique transcriptomic and metabolic responses to protozoan predation. Next to defense against competitors and predators, LPs are well-known for their role in swarming motility, a flagella-driven multicellular behavior of bacteria. Orfamide-deficient mutants of *P. protegens* Pf-5, either with deletions in the biosynthesis gene *ofaA* or in the regulatory gene *gacA*, cannot swarm on their own but 'hitch-hike' with parental strain Pf-5. However, distinctly different spatial distributions in co-swarming colonies were observed for these two mutants, with the *ofaA* mutant moving behind the wild type and the *gacA* mutant predominating on the edge of the swarming colony. Subsequent experimental evolution assays showed that repeated swarming cycles of strain Pf-5 drives parallel evolution toward fixation of spontaneous *gacS/gacA* mutants on the edge, ultimately

causing colony collapse. Transcriptome analyses revealed that genes associated with resource acquisition, motility, chemotaxis and efflux were significantly upregulated in these regulatory mutants. Moreover, microscopic analysis showed that *gacA* mutant cells were longer and more flagellated than wild type and *ofaA* mutant cells, which may explain their predominance on the edge of co-swarming colonies. Collectively, these results indicated that adaptive convergent evolution through point mutations is a common feature of range-expanding microbial populations and that the putative fitness benefits of these spontaneous mutations during dispersal of bacteria into new territories are frequency-dependent.

Samenvatting

Cyclische lipopeptiden (LPs) zijn oppervlakte-aktieve en antibiotische metabolieten die zijn opgebouwd uit een vetzuur gekoppeld aan een kort lineair of cyclisch oligopeptide. In bacteriën worden LPs gesynthetiseerd door nonribosomale peptide synthetases (NRPS). Ondanks dat de biosynthese van LPs al uitvoerig is onderzocht en beschreven is er nog relatief weinig bekend over hoe de biosynthese van LPs gereguleerd wordt. **Het doel van dit proefschrift** was om genen betrokken bij de regulatie van LP biosynthese te identificeren en om de natuurlijke functies van LPs geproduceerd door plant-geassocieerde *Pseudomonas* bacteriën te ontrafelen. Door de combinatie van verschillende 'omics'-gebaseerde technieken hebben wij twee RNAs, genaamd RsmY en RsmZ, geïdentificeerd die samen met de repressor eiwitten RsmA en RsmE de biosynthese van massetolide reguleren, een LP geproduceerd door de rhizosfeerbacterie *Pseudomonas fluorescens* SS101. Tevens werden, met behulp van het aanbrengen van random mutaties in het genoom *Pseudomonas fluorescens* SS101, vier andere regulatie-genen (*phgdh*, *dnaK*, *prtR* and *clpA*) geïdentificeerd. Mutaties in elk van deze genen resulteerden in een verlies van massetolide productie, alsmede in een verlies van motiliteit en biofilmvorming, twee eigenschappen waar LPs een belangrijke rol in spelen. Ook hebben we aangetoond dat massetolide biosynthese gereguleerd wordt door het ClpAP protease complex via de transcriptionele regulator MassAR, de heat-shock eiwitten DnaK en DnaJ alsmede specifieke eiwitten betrokken bij de citroenzuurcyclus.

LPs hebben antibiotische activiteit en diverse andere natuurlijke functies voor de producerende bacteriën. LPs van *P. fluorescens* spelen onder andere een grote rol in de verdediging tegen predatie door protozoa. Transcriptoom analyses toonden aan dat 55 genen verhoogd en 73 genen verlaagd tot expressie kwamen in *P. fluorescens* SS101 in aanwezigheid van *Naegleria americana*, een protozoa die zich voedt met bacteriën. Naast de massetolide biosynthese genen *massABC* kwamen ook de genen betrokken bij alkaandegradatie en putrescine catalyse verhoogd tot expressie. Putrescine induceerde cyst–vorming in de protozoa, wat mogelijk een tweede lijn van verdediging tegen predatie biedt. Met behulp van MALDI Imaging Massa Spectrometrie (IMS) en live colony NanoDesi Massa Spectrometrie werd de productie van massetolide in de interactie-zone tussen de bacteriën en de protozoa zichtbaar gemaakt. Vergelijkbare transcriptionele en metabole veranderingen werden waargenomen in experimenten met de verwante stam *P. fluorescens* SBW25, maar er werden ook stam-specifieke reacties gevonden. Deze resultaten laten zien dat zeer verwante bacteriestammen gelijke maar ook unieke transcriptionele en metabole reacties geven op predatie door protozoa.

Naast een rol in de verdediging tegen predatie en in competitie, zijn LPs zeer bekend vanwege hun rol in zwermen, een bewegingsvorm van bacteriën waarbij de cellen voortgestuwd worden door flagellen oftewel zweepstaarten. Mutanten van *P. protegens* Pf-5 met mutaties in het LP-biosynthese gen *ofaA* of het LP-regulatie gen *gacA* zijn beide deficient in de productie van het LP orfamide. Deze twee mutanten kunnen niet meer

zelfstandig zwermen maar kunnen wel ‘meeliften’ met het LP-producerende wildtype Pf-5. Echter, uit onderzoek naar de ruimtelijke verdeling van deze twee mutanten tijdens het meeliften bleek dat de *ofaA* mutant het wildtype volgde terwijl de *gacA* mutant zich voor het wildtype bevond aan de rand van de zwermende kolonie. Verdere experimenten lieten zien dat gedurende opeenvolgende cycli van zwermende Pf-5 cellen er spontane *gacS*/*gacA* mutanten accumuleren aan de rand van de kolonie, wat er uiteindelijk voor zorgde dat de kolonie zich niet meer kon voortbewegen. Transcriptoom analyses van deze spontane *gacS*/*gacA* mutanten lieten tevens zien dat genen geassocieerd met nutriënten acquisitie, motiliteit, chemotaxis en efflux verhoogd tot expressie komen. Microscopische analyses toonden verder aan dat de cellen van deze spontane mutanten langer zijn en meer flagellen bevatten dan wildtype Pf-5 of de *ofaA* mutant. Dit kan mogelijk een reden zijn waarom de *gac* mutanten zich voornamelijk aan de rand van de zwermende kolonies bevinden. Samengevat wijzen deze resultaten erop dat adaptieve convergente evolutie door specifieke puntmutaties een algemeen kenmerk is van zwermende bacteriën en dat deze puntmutaties mogelijk een positieve bijdrage kunnen leveren aan de fitness tijdens verspreiding van bacteriën naar nieuwe territoria.

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Curriculum vitae

Chunxu Song (宋春旭) was born on the 27th of November, 1983 in Beijing, China. She started her BSc study in Biotechnology at Huazhong Agricultural University, Wuhan, China in 2001. During the BSc period, she also obtained a minor degree in English. In 2005, she continued with her MSc study in Biochemistry and Molecular Biology at Huazhong Agricultural University. For her MSc thesis, she did research on antibiotics produced by *Bacillus thuringiensis* (Bt) strains which are commonly used as biological pesticide in agriculture, under the supervision of Prof. Ming Sun. In 2008, she started her PhD project entitled "Regulation and natural function of lipopeptide biosynthesis in *Pseudomonas*" in the Laboratory of Phytopathology at Wageningen University, under the supervision of Prof. Jos Raaijmakers and Prof. Francine Govers. Since May 2013, she has been working as a Postdoc on comparative genomics and genome mining for antimicrobial compounds of *Collimonas* strains in the Laboratory of Microbial Ecology at the Netherlands Institute of Ecology (NIOO-KNAW).

Publications

C. Song*, M. Mazzola*, X. Cheng, T. Alexandrov, J. Oetjen, P. Dorrestein, J. Watrous, M. van der Voort & J.M. Raaijmakers. Molecular and chemical dialogues in bacteria-protozoa interactions. Submitted.

C. Song, T.A. Kidarsa, J.E. van de Mortel, J.E. Loper & J.M. Raaijmakers. Living on the edge: spatial heterogeneity and convergent evolution of social cheaters in swarming colonies of *Pseudomonas protegens*. Submitted.

C. Zachow, G. Jahanshah, I. de Bruijn, **C. Song**, F. Ianni, Z. Pataj, H. Gerhardt, I. Pianet, M. Lämmerhofer, G. Berg, H. Gross, J.M. Raaijmakers (2015). The novel lipopeptide poaeamide of the endophyte *Pseudomonas poae* RE*1-1-14 is involved in pathogen suppression and root colonization. **Molecular Plant-Microbe Interactions**. Accepted.

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* Those authors contributed equally.

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: Chunxu Song
Date: 24 April 2015
Group: Laboratory of Phytopathology
University: Wageningen University & Research Centre

1) Start-up phase	*date*
► **First presentation of your project**	
Regulation of cyclic lipopeptide biosynthesis in beneficial bacteria and the role of plant seed exudates	Jun 05, 2009
► **Writing or rewriting a project proposal**	
Writing a review or book chapter	
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*1.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
PhD student day, Leiden University	Febr 26, 2009
PhD student day, Utrecht University	Jun 01, 2010
► **EPS theme symposia**	
EPS theme 2 symposium 'Interaction between Plants and Biotic Agents', Utrecht University	Jan 22, 2009
EPS theme 2 symposium 'Interactions between Plants and Biotic Agents', Utrecht University	Jan 15, 2010
EPS theme 2 symposium and Willie Commelin Scholten Day, Wageningen University	Feb 10, 2012
► **NWO Lunteren days and other National Platforms**	
ALW Platform Molecular Genetics Annual Meeting, Lunteren	Oct 15-16, 2009
ALW Platform Molecular Genetics Annual Meeting, Lunteren	Oct 06-07, 2011
► **Seminars (series), workshops and symposia**	
Scientific Spring Meeting NVMM & NVvM 2009, Arnhem	Apr 22, 2009
Seminar Dr. Rays H.Y. Jiang	Jun 10, 2009
EPS symposium "Ecology and Experimental Plant Sciences 2", Wageningen	Sep 22, 2009
Plant Sciences Seminar Prof. Pierre de Wit and Prof. Fred van Eeuwijk	Nov 10, 2009
Plant Sciences Seminar Prof. Olaf van Kooten and Prof. Jack Leunissen	Nov 13, 2009
Seminar Richard Oliver	Nov 20, 2009
Seminar Prof. Nick Panopoulos	Jan 11, 2010
Seminar Laurent Zimmerli	Jan 25, 2010
Plant Sciences Seminar Prof. Holger Meinke and Prof. Paul Struik	Apr 13, 2010
The Omics Promise: Opportunities for Environmental Objectives, Bilthoven	Apr 15, 2010
Plant Sciences Seminar Prof. Louise Vet and Just Vlak	May 11, 2010
Wageningen Evolution and Ecology Seminars Toby Kiers	Jun 17, 2010

	Seminar Prof. Naoto Sibuya	Sep 09, 2010
	Seminar Prof. David Baulcombe	Sep 27, 2010
	Seminar Dr. Kirsten Bomblies	Nov 18, 2010
	1st meeting WUR-NIOO Centre for Soil Ecology, Wageningen	Jun 27, 2011
	Seminar Rosie Bradshaw	Aug 04, 2011
	Birgit Piechulla : The smell of rhizobacteria: biological and chemical aspects	Oct 04, 2012
	Gabriele Berg: Plant microbes: Specificity and impact on plant health	Oct 09, 2012
	Talent day for women scientists - Pump your Career 2012	Oct 11, 2012
	WEES seminar Bertus Beaumont: Adaptive radiation, flagella and the evolution of biological complexity	Jan 24, 2013
	Evolution in the laboratory	Mar 14, 2013
	WEES seminar David Berry: Ecological and evolutionary aspects of the gut microbiota in health and inflammation	Mar 21, 2013
	Seminar Kathrin Riedel: Metaproteomics - novel insights into old questions in medical microbiology and microbial ecology	Mar 25, 2013
	WEES seminar Martin Ackermann: An evolutionary perspective on bacterial individuality	Jun 20, 2013
	'Last Stretch of the PhD Programme' workshop	Aug 02, 2013
	Seminar Dr. Pieter Dorrestein: A "GoogleMAP"-type molecular view of microbes - from culture to people	Aug 22, 2013
	Seminar Daniel Rozen	Sep 09, 2013
	Semiar Sara Mitri: Modelling the evolution of competition and cooperation between strains and species of bacteria	Sep 24, 2013
	Ross Mann: Endophytes in agriculture - evaluating their application via metabolomics and genomics	Oct 04, 2013
	Seminar Gabriele Berg	Oct 14, 2013
	PacBio Seminar	Mar 26, 2014
	EPS Symposium 'Omics Advances for Academia and Industry - Towards True Molecular Plant Breeding'	Dec 11, 2014
►	**Seminar plus**	
►	**International symposia and congresses**	
	Annual Ecogenomics meeting 2009, Amsterdam	Apr 16-17, 2009
	FEMS meeting, Gothenburg, Sweden	Jun 29-Jul 02, 2009
	2012 PhD retreat in Norwich, UK	Aug 14-17, 2012
	15th International Symposium on Microbial Ecology (ISME meeting)	Aug 24-29, 2014
►	**Presentations**	
	Poster in FEMS meeting: Genome-wide identification of genes involved in the regulation of cyclic lipopeptide biosynthesis in Pseudomonas fluorescens	Jul 01, 2009
	Oral presentation: PhD summer school: Rhizosphere Signaling: Regulation of cyclic lipopeptide biosynthesis in Pseudomonas fluorescens	Aug 23-25, 2010
	Oral presentation: ALW Platform Molecular Genetics: Genetics and evolution of swarming motility in Pseudomonas	Oct 06-07, 2011
	Oral presentation: EPS Autumn School 2011 "Host-Microbe Interactomics": The role of lipopeptides in Bacteria-Protozoa interactions	Nov 01-03, 2011
	Oral presentation: PhD retreat: Regulation and natural functions of lipopeptide biosynthesis in Pseudomonas fluorescens	Aug 14-17, 2012
	Oral presentation EEDG meeting: Living on the edge: short-term evolution in swarming bacteria	May 31, 2013

	Oral presentation PhD meeting: Regulation and natural functions of cyclic lipopeptide biosynthesis in Pseudomonas fluorescens	Aug 08, 2013
	Oral presentation NIOO meeting: Regulation and natural functions of cyclic lipopeptide biosynthesis in Pseudomonas strains	Sep 09, 2013
	Oral presentation ISME: Living on the edge: spatial heterogeneity and convergent evolution of social cheaters in swarming colonies of Pseudomonas protegens	Aug 28, 2014
►	**IAB interview**	
	Meeting with a member of the International Advisory Board of EPS	Nov 15, 2012
►	**Excursions**	
	Rijk Zwaan excursion	Sep 27, 2013
	Subtotal Scientific Exposure	*21.8 credits**

3) In-Depth Studies		*date*
►	**EPS courses or other PhD courses**	
	Information Literacy PhD including EndNote Introduction	Apr 07-08, 2009
	Bioinformatics: A Users Approach	Mar 15-19, 2010
	Summer school Rhizosphere signaling	Aug 23-25, 2010
	EPS Autumn School 2011 “Host-Microbe Interactomics”	Nov 01-03, 2011
	Introduction to R course	May 19-20, 2014
►	**Journal club**	
	Monday afternoon literature discussion in Bacteria Ecology & Genomics group, Phytopathology	2008-2013
►	**Individual research training**	
	Proteomic analysis in Sweden	Feb 2011
	Subtotal In-Depth Studies	*8.4 credits**

4) Personal development		*date*
►	**Skill training courses**	
	Presentation Skills	Oct 06-10, 2009
	Academic Writing II	Sep 2009-Feb 2010
	PhD expectation day	Nov 19, 2010
	KLV Fast reading	Sep 15, 2010
	Scientific Writing	Apr 20-Jun 08, 2011
	KLV Guidelines for finding a job in NL	Sep 07, 2011
	KLV CV writing	Sep 23, 2011
	EPS Career Day	2012
	Career Orientation	Sep-Oct 2012
	PCDI Postdoc Retreat 2015 - Life Sciences	Mar 25-27, 2015
►	**Organisation of PhD students day, course or conference**	
	2010 Organizing labouting	May 27, 2010
►	**Membership of Board, Committee or PhD council**	
	Subtotal Personal Development	*10.5 credits**

TOTAL NUMBER OF CREDIT POINTS*	**42.2**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

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