



## Bioactivity and phylogeny of the marine bacterial genus *Pseudoalteromonas*

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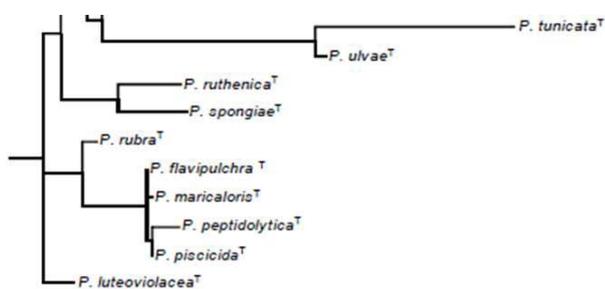
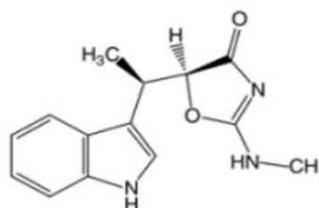
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# Bioactivity and phylogeny of the marine bacterial genus *Pseudoalteromonas*

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Ph.D. thesis

by

Nikolaj Grønnegaard Vynne

2011

Technical University of Denmark

National Food Institute

Division of Industrial Food Research

Cover illustrations:

Map of the Galathea 3 route ([www.galathea3.dk](http://www.galathea3.dk))

Structure of the antibiotic indolmycin

Excerpt of 16S rRNA phylogenetic tree

*P. luteoviolacea* S4054 grown in MMM

## PREFACE

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The present Ph.D. study has been conducted at the National Food Institute (previously the National Institute for Aquatic Resources) at the Technical University of Denmark. The study lasted from January 2009 to December 2011 under the supervision of Professor Lone Gram. The project included a 12 week research stay with Professor Debra Milton at the Department of Molecular Biology, Umeå University, Sweden.

The Ph.D. project was carried out as part of the collaborative research project “Discovery of novel bioactive bacteria and natural products and their use to improve human health and safety” funded by the Programme Commission on Health, Food and Welfare under the Danish Council for Strategic Research.

The work resulted in the preparation of four manuscripts which form the basis of this thesis. In addition, one genome announcement is in preparation for the Journal of Bacteriology, and collaborative efforts within the frame of the research project will likely result in a co-authorship (not included in this thesis).

Nikolaj G. Vynne

December 2011

## SUMMARY

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The purpose of this Ph.D. project was to evaluate a global collection of marine *Pseudoalteromonas* bacteria as a source of novel bioactive compounds, and to investigate the distribution and production of such compounds among different species within the *Pseudoalteromonas* genus. The strain collection was obtained during the research cruise “Galathea 3”, which circumnavigated the Earth while screening marine bacteria for the ability to inhibit *Vibrio anguillarum* 90-11-287. *Pseudoalteromonas* strains were one of the most frequently isolated genera.

The *Pseudoalteromonas* strains were evaluated for their ability to repeatedly inhibit the fish pathogen *Vibrio anguillarum* 90-11-287 or *Staphylococcus aureus* 8325. Based on previous work, a hypothesis that antagonistic *Pseudoalteromonas* strains primarily were pigmented and surface associated was investigated. This Ph.D. work confirmed that surface-associated strains were significantly more likely to possess stable antibacterial activity and be pigmented. *Pseudoalteromonas* strains are known as prolific producers of bioactive secondary metabolites; hence screening the global strain collection for production of novel antibiotics was initiated. Novel quinolone-related compounds were described, but were not antibacterial. Several antibacterial compounds known from other sources were identified, for instance indolmycin which was hitherto only known from terrestrial *Streptomyces*. Genome sequencing of *P. luteoviolacea* S4054 revealed up to 11 biosynthetic pathways with unknown products, confirming the potential for discovery of new secondary metabolites from *Pseudoalteromonas* strains.

The elaborate secondary metabolite production led me to speculate whether it was possible to use secondary metabolites to assist in species identification within this genus. This would also provide information on the use of 16S rRNA gene sequences to dereplicate strain collections in biodiversity efforts. A phylogenetic study of 16S rRNA gene sequences of the *Pseudoalteromonas* strains confirmed the division into two clades; one consisted of bioactive pigmented strains and one predominantly of inactive non-pigmented strains. Correlating this to a dendrogram based on the secondary metabolites in each strain showed that some strains clustered together in a species-specific way, whereas other strains did not cluster near strains of the same species. Hence, secondary metabolite production was not unequivocally reflected in the secondary metabolite profile, possibly due to the limited resolving power of the 16S rRNA gene. The species *P. luteoviolacea* showed an interesting pattern indicative of phylotype specific antibiotic production strains. Detailed phylogenetic analysis of an expanded collection of *P. luteoviolacea* strains showed confirmed that production of antibiotics was related to phylogeny within this species, which indicates that

the underlying biosynthetic pathways are maintained under selective pressure and hence are important traits for the organism.

One strain stood out during work with the strain collection, in part because of its production of an intense black pigment in contrast to its phylogenetic placement within the non-pigmented clade. This strain was subsequently shown to represent a new bacterial species named *Pseudoalteromonas galathea*.

Initial studies revealed the potential production of regulatory compounds involved in cell to cell signaling within some strains of the species *P. luteoviolacea*. Since such mechanisms are known to govern antibiotic production in some bacteria, this was investigated. A quorum sensing system controlling a putative novel biosynthetic pathway with high homology to the *lux* system of *Vibrio fischeri* was identified in *P. luteoviolacea* S4054. The signal molecule was potentially a new acylated homoserine lactone (AHL) like compound, and the AHL synthetase was phylogenetically distinct from related synthetases. This expands our knowledge of bacterial signaling and *lux* homologue system, and further work will resolve if this system has implications for antibiotic production.

In summary, this Ph.D. work explored the phylogeny and chemical diversity of the genus *Pseudoalteromonas*. Novel compounds were discovered but they possessed no antibiotic activity. However, analysis of the genome sequence of *P. luteoviolacea* S4054 revealed genetic potential for discovery of secondary metabolites not known within this species. Secondary metabolites were not unequivocally representative of species assignments, but on an intra-species level the use of detailed phylogenetic analysis showed phylotype specific production of antibiotics within the species *P. luteoviolacea*. These findings validate the genus *Pseudoalteromonas* as a potential source of novel secondary metabolites and may be useful when designing future biodiscovery strategies. The novel species *P. galathea* was described which contributed to resolving the taxonomy of the genus. This thesis also provides evidence of a quorum sensing system related to the *lux* system of *Vibrio fischeri* but relying on putatively novel signaling molecule encoded by a distinct synthetase, which might be involved in the regulation of antibiotics production.

## RESUMÉ

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Formålet med dette Ph.D. studie var at udforske det bioaktive potentiale af indsamlede marine *Pseudoalteromonas* bakterier, samt at kortlægge hvorledes produktionen af bioaktive stoffer er fordelt imellem de enkelte *Pseudoalteromonas* arter. Stammesamlingen blev grundlagt under en jordomsejling med forskningsekspeditionen "Galathea 3", hvor marine bakterier blev screenet for hæmmende effekt mod *Vibrio anguillarum* 90-11-287. *Pseudoalteromonas* stammer udgjorde en af de primære slægter, der blev isoleret.

*Pseudoalteromonas* stammerne blev testet for hæmmende effekt mod *Vibrio anguillarum* 90-11-287 og *Staphylococcus aureus* 8325. Baseret på tidligere arbejder udviklede jeg den hypotese at *Pseudoalteromonas* stammer der udviser en stabil hæmmende effekt oftere er pigmenterede eller isoleret fra et overfladeskrab. Det blev bekræftet via en statistisk analyse af de indsamlede bakterier. Det er kendt at *Pseudoalteromonas* stammer producerer bioaktive sekundære metabolitter, og på den baggrund igangsatte jeg en screening af de indsamlede *Pseudoalteromonas* stammer for at identificere nye stoffer med antibakteriel aktivitet. Denne screening resulterede i identifikation af ukendte quinolon-relatede stoffer, men de var ikke antibakterielle. Derimod blev der isoleret allerede kendte antibiotika, f.eks. indolmycin som hidtil udelukkende var fundet i *Streptomyces* bakterie isoleret fra jord. Genomsekventering af *P. luteoviolacea* S4054 afslørede op til 11 biologiske pathways med ukendte slutprodukter der kunne være involverede i syntesen af sekundære metabolitter, hvilket bekræfter potentialet for opdagelse af nye stoffer fra *Pseudoalteromonas* bakterier.

Den omfattende produktion af sekundære metabolitter blandt *Pseudoalteromonas* stammerne førte til den ide at de sekundære metabolitter måske kunne bruges som et redskab til at adskille de forskellige arter inden for slægten. Dette ville også afklare om 16S rRNA gensekvenser kan bruges som et redskab til dereplikering af bakteriestammer i forbindelse med screening for sekundære metabolitter. En fylogeni baseret på 16S rRNA gensekvenser viste, som ventet baseret på tidligere studier, at stammerne opdeltes i to grupper. Den ene gruppe indeholdt hæmmende pigmenterede stammer, mens den anden gruppe primært indeholdt ikke-pigmenterede stammer uden hæmmende aktivitet. Ved sammenligning med et dendrogram baseret på produktion af sekundære metabolitter kunne det konstateres at nogle arter havde en ensartet sekundær metabolitprofil, mens andre arter producerede forskellige sekundære metabolitter alt efter hvilken stamme der blev undersøgt. Derfor kunne 16S rRNA gensekvenser ikke bruges som pålidelig metode til dereplikation af bioaktive *Pseudoalteromonas* stammer. Arten *P. luteoviolacea* udviste dog et interessant mønster der tydede på en overensstemmelse med fylogenetisk opdeling og

produktion af sekundære metabolitter. En detaljeret fylogenetisk analyse baseret på recombinase A og gyrase B generne afslørede at produktionen af to forskellige antibiotika var begrænset til hver sin fylogenetiske gruppering. Dette tyder på at generne der er involveret i biosyntesen af disse antibiotika bliver bevaret under et selektivt pres, som betyder at antibiotikaproduktionen er et vigtigt træk for organismen.

Under arbejdet med stammesamlingen stod én stamme ud på grund af dens karakteristiske sorte pigment, som den producerede på trods af at fylogenetisk analyse placerede stammen i den ikke-pigmenterede gruppe. Denne stamme skulle vise sig at repræsentere en ny bakterieart som blev navngivet *Pseudoalteromonas galathea*.

Indledende studier viste, at visse *P. luteoviolacea* stammer producerede stoffer, der potentielt var involveret i kommunikation imellem cellerne. Sådanne mekanismer regulerer produktionen af antibiotika i visse andre bakterier, og blev derfor undersøgt nærmere i *P. luteoviolacea* S4054. Et quorum sensing system med stor lighed til *lux* systemet fra *Vibrio fischeri* blev identificeret. Selve signalmolekylet tegner til at være en ukendt acyleret homoserin lakton (AHL) analog, og i en fylogenetisk undersøgelse adskilte AHL syntasen Plul sig tidligt fra de nærmest beslægtede proteiner fra *Vibrio* bakterier, hvilket tyder på at det er en hidtil ukendt AHL syntase. Dette styrker vor viden om bakterielle signalsystemer, og videre arbejde vil klarlægge hvorvidt dette system har betydning for antibiotikaproduktionen i *P. luteoviolacea* S4054.

For at opsummere har dette Ph.D. studie udforsket fylogeni og kemisk diversitet i slægten *Pseudoalteromonas*. Ingen nye antibiotika blev identificeret, men nye stoffer blev opdaget og det genetiske potentiale af *P. luteoviolacea* S4054 tyder Sekundære metabolitter var ikke universelt brugbare til artsidentifikation, men inden for arten *P. luteoviolacea* afspejledes produktionen af antibiotika i den fylogenetiske opdeling. Disse opdagelser tyder på at *Pseudoalteromonas* stammer er lovende mål for opdagelse af hidtil ukendte stoffer med biologisk aktivitet, og detaljeret fylogenetisk opklaring kan bruges som et værktøj i opdagelsen af sådanne nye stoffer. Den nye art *P. galathea* blev beskrevet hvilket bidrog til en afklaring af taxonomien inden for slægten *Pseudoalteromonas*. Under dette Ph.D. arbejde blev det opdaget at *P. luteoviolacea* S4054 besad et quorum sensing system, der potentielt regulerer produktionen af antibiotika. Quorum sensing systemet havde ligheder med det *lux* system, der kendes fra *V. fischeri*, men de enkelte komponenter var ikke fuldt ud identiske og repræsenterer muligvis nye varianter af AHL syntase og AHL signal molekyler.

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## LIST OF ARTICLES

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- 1 Vynne N, Månsson M, Nielsen KF, Gram L (2011) Bioactivity, chemical profiling, and 16S rRNA-based phylogeny of *Pseudoalteromonas* strains collected on a global research cruise. *Mar. Biotechnol.* 13: 1062-1073.
- 2 Vynne N, Månsson M, Nielsen KF, Gram L (2012) Antibiotic chemoprofile and RecA and GyrB sequence analyses reveal three phylotypes within the marine *Pseudoalteromonas luteoviolacea*. Manuscript submitted to *Applied and Environmental Microbiology*.
- 3 Vynne N, Christiansen G, Gram L (2012) *Pseudoalteromonas galathea* S3431 sp. nov., a novel *Pseudoalteromonas* species isolated from a deep-sea polychaete. Manuscript in preparation.
- 4 Vynne N, Månsson M, Nielsen KF, Milton D, Gram L (2012) Production of a putatively novel acylated homoserine lactone-like quorum sensing activating molecule by *Pseudoalteromonas luteoviolacea* S4054. Manuscript in preparation.

### Non peer reviewed publications:

Vynne N, Gram L (2012) Draft genome sequence of *Pseudoalteromonas luteoviolacea* S4054, an indolmycin producing marine bacterium. In preparation.

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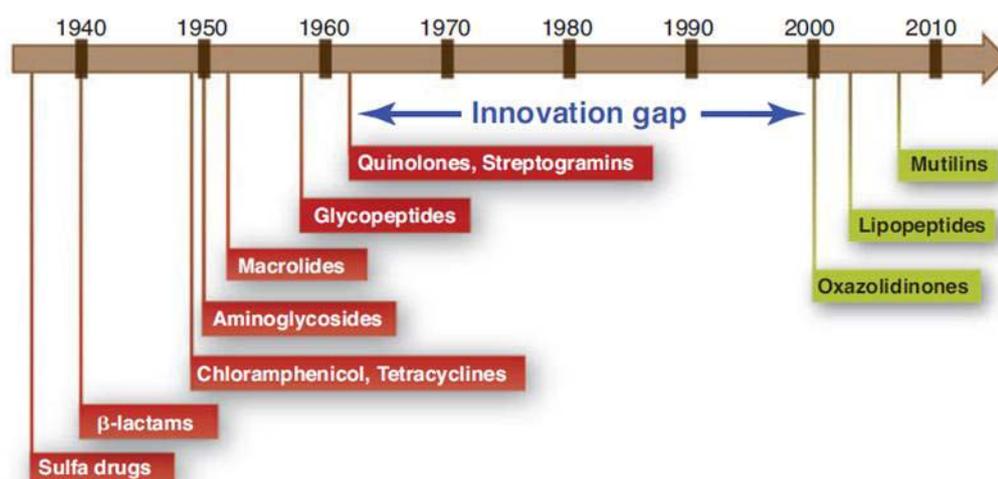
National Food Institute

Division of Industrial Food Research

## 1.0 INTRODUCTION AND BACKGROUND OF THE PH.D. PROJECT

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The increase in hospital and community acquired infections caused by drug resistant bacteria is currently making an impact on societies worldwide. Developing new antibiotics is of the outmost importance, and searching for antibiotics that have a novel mechanism of action or are structurally distinct from those currently in use is one strategy to combat the spread of existing resistance mechanisms. Unfortunately, a combination of factors led to a decline in development of antibiotics, beginning in the late 1960's and lasting almost 40 years (Fischbach & Walsh, 2009). In this so called innovation gap, no new classes of antibiotics were introduced (figure 1). In fact, 73% of the approved antibiotics between 1981 and 2005 belonged to just three antibiotic classes:  $\beta$ -lactams, macrolides and quinolones (Hughes & Fenical, 2010; Newman & Cragg, 2007).



**Figure 1:** An illustration of the innovation gap from 1962 to 2000. No new classes of antibiotics were developed, leading to an almost empty drug pipeline when resistance against known antibiotic classes emerged. Adapted from Fischbach *et al.* (2009).

Part of the reason for this lack of new antibiotic classes is the failure of combinatorial chemistry to deliver a significant number of lead compounds to the pharmaceutical development pipeline (Newman & Cragg, 2007) and the low profitability of developing antibiotics compared to classic blockbuster drugs within e.g. cancer or metabolic conditions (Malik, 2008; Nathan & Goldberg, 2005).

The marine environment is considered an underexplored source of novel bioactive natural products (Fenical, 1993; Gulder & Moore, 2009; Imhoff *et al.*, 2011; Molinski *et al.*, 2008) and the structural diversity of marine natural products (Newman & Cragg, 2007) make them a promising target for discovery of novel antibiotics. The potential for discovery of structurally diverse novel antibiotics is enormous considering oceans cover 70% of Earth and even the marine Polar Regions are teeming with life. Antagonistic compounds are widely believed to play important roles in microbial ecology, for instance through chemical warfare among bacteria or as weapons in prey-predator interactions. The dual role of antibiotics in human therapeutics and microbial interactions is fascinating, and we are only just beginning to understand the context of this within research areas such as development of antibiotic resistance, microbial signaling, bacteria-eukaryote interactions and microbial community dynamics.

During the research expedition 'Galathea 3', the project 'Stars of the ocean' isolated antagonistic marine bacteria. The majority of the isolated bacterial strains were identified as species of *Vibrio*, *Pseudoalteromonas* or the *Roseobacter* clade. (Gram *et al.*, 2010). A subsequent grant from the Strategic Research Council for Food, Health and Welfare allowed the establishment of the project "Discovery of novel bioactive bacteria and natural products and their use to improve human health and safety" involving two research groups from the Technical University of Denmark (DTU) and three from University of Copenhagen (KU). The main research areas were divided, with work at the National Food Institute focused on cultivation and ecology of marine bacteria and screening for antagonistic compounds, DTU Systems Biology focused on purification and structure elucidation of bioactive compounds, KU-LIFE focused on virulence inhibition in *Staphylococcus aureus* by marine natural products and interactions of marine bacterial metabolites with the human immune system, and KU-Health focused on marine natural products in interference with quorum sensing.

### 1.0.1 SCOPE OF THE PH.D. PROJECT

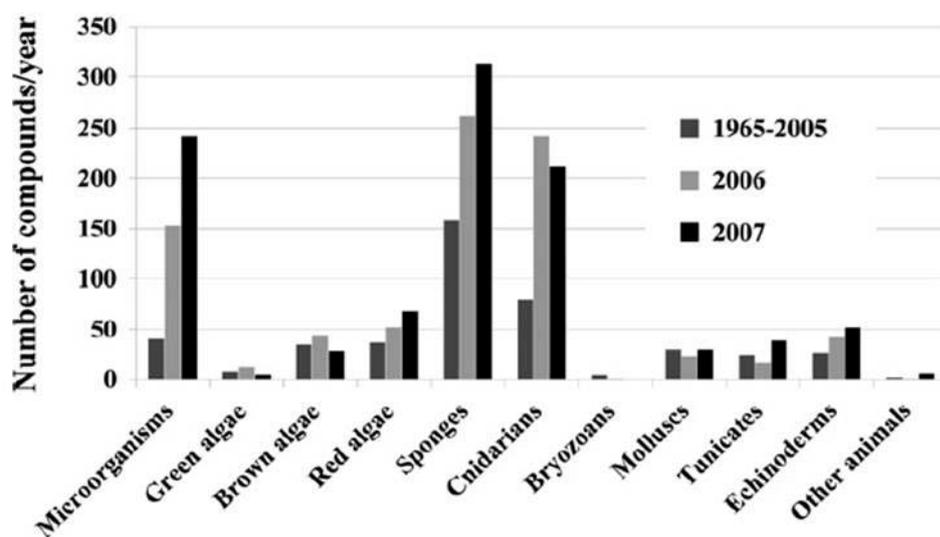
This Ph.D. work targeted the group of strains isolated during the ‘Galathea 3’-expedition which belong to the marine *Pseudoalteromonas* genus. Pseudoalteromonads are known to frequently be bioactive (Bowman, 2007), and one of my primary aims was to screen the global collection of *Pseudoalteromonas* strains for antagonistic activity in order to isolate novel antibiotics. Bioactive *Pseudoalteromonas* strains are often found in association with higher eukaryotes or marine surfaces (Holmström & Kjelleberg, 1999), and we observed that production of antibacterial compounds often co-occurred with pigmentation. The nature of the strain collection allowed me to address the hypothesis that antagonistic *Pseudoalteromonas* are predominantly pigmented and surface associated (Vynne *et al.*, 2011) using statistical tools. The bioactive potential of Pseudoalteromonads is well known (Bowman, 2007), hence screening a diverse global collection of *Pseudoalteromonas* strains for production of novel secondary metabolites might result in interesting compounds with e.g. antibiotic activity and provide an overview of the chemical diversity of the genus. Additionally, I hypothesized that secondary metabolites might be useful taxonomic markers to assist in species discrimination among *Pseudoalteromonas* strains since the majority of bioactive strains form a distinct clade when subjected to phylogenetic analysis of 16S rRNA gene sequences (Gauthier *et al.*, 1995). This might also facilitate biodiscovery strategies by providing an overview of the distribution of compounds among *Pseudoalteromonas* species. In working with the strain collection, one strain stood out in particular due to its production of an intense black pigment. This strain was identified as a new species; *Pseudoalteromonas galathea* (Vynne *et al.*, 2012a). During the screening for novel antibiotics I observed that four *P. luteoviolacea* strains based 16S rRNA phylogeny divided into two groups, each producing the common bioactive violacein and a specific antibiotic for the phylogenetic group in question. Based on these observations and studies of species specific production of secondary metabolites in the Actinomycete genus *Salinispora* (Jensen *et al.*, 2007), I developed a hypothesis that phylogenetic reconstruction of the evolutionary relationship among *Pseudoalteromonas* strain could be used to guide biodiscovery efforts at the sub-species level and avoid characterization of overlapping secondary metabolite profiles. This I showed within the species *Pseudoalteromonas luteoviolacea* (Vynne *et al.*, 2012b). The strain *P. luteoviolacea* S4054 produced molecules capable of activating several quorum sensing monitor strains. The presence of a putative cell-density dependant

signaling system could suggest that one or more interesting phenotypes such as antibiotic production were controlled in this manner, which I investigated experimentally. The work on cell signaling is not yet concluded, however I have shown that a signaling system is present in *P. luteoviolacea* and that it might regulate a novel biosynthetic pathway (Vynne *et al.*, 2012c). The signaling molecules are AHL-like and may also be novel. Finally, the potential for secondary metabolite production was assessed based on genome analysis, which indicated that strain S4054 has the potential to produce several so far unknown compounds (Vynne & Gram, 2012).

This Ph.D. thesis contains an overview of drug discovery strategies within marine bacteria and how efforts in microbial ecology and drug discovery may benefit from interacting (chapter 2). Chapter 3 describes the ecology, industrial applications and biodiscovery potential of the marine bacterial genus *Pseudoalteromonas*, and chapter 4 presents an overview of quorum sensing and the relevance of quorum sensing in drug discovery.

## 2.0 BIOACTIVE SECONDARY METABOLITES FROM MARINE BACTERIA

Bacterial secondary metabolites have historically played a central role in discovery of novel pharmaceutical lead compounds. In particular, terrestrial Actinobacteria have been a major source of biologically active secondary metabolites and most antibiotics in use today are either natural products or natural product derived (Newman & Cragg, 2007). Among the success stories are the discovery of streptomycin and tetracycline, with tetracycline and synthetic derivatives still being backbone drugs in treatment of bacterial infections when resistance is not present. Recent years have seen a dramatic increase in the discovery rate of marine natural products, many of which originate from marine microorganisms (figure 2). As we learn that marine eukaryotes may harbor distinct and species specific bacterial epibionts, it is becoming evident that some chemical compounds previously attributed to the macroorganism are in fact produced by epibiont bacteria. The discovery and origin of bryostatins, currently undergoing clinical trials as an anti-cancer agent, exemplifies this. Bryostatin 1, currently in phase I clinical trials against cancer, was originally isolated from samples of the marine bryozoan *Bugula neritina* and was immediately recognized for



**Figure 2:** Number of new marine natural compounds discovered broken down by phylum. The compounds discovered from 1965 to 2005 were summed up and averaged. Adapted from Blunt *et al.* (2009)

its potent anticancer activity (Pettit *et al.*, 1982). Initially thought to be a product of the bryozoan animal, it was later revealed that the biosynthetic genes were actually present in the bacterial symbiont “*Candidatus Endobugula sertula*” (Sudek *et al.*, 2006) but not in the bryozoan. Production of bryostatins was also reduced after an antibiotic treatment and accompanying reduction in the number of bacterial symbionts (Sudek *et al.*, 2006) offering strong evidence that the bryostatins are in fact of bacterial origin. The marked increase in discovery rates of microbially produced marine natural products likely in part due to an increased awareness of microbial epibionts as potential sources of bioactive metabolites (Mearns-Spragg *et al.*, 1998; Penesyan *et al.*, 2009; Sudek *et al.*, 2006), but it is also an acknowledgement of the importance of marine microorganisms as a promising source of biologically active molecules. In 2010 the marine drugs pharmaceutical pipeline contained twelve drugs in phase I to III clinical trials (table 1) mainly targeting cancer, and four approved drugs (Mayer *et al.*, 2010).

**Table 1:** The status of the marine pharmaceutical pipeline, 2010. Modified from Mayer *et al.* (2010).

Clinical status	Compound name	Chemical class	Marine organism	Disease area
Approved	Cytarabine, Ara-C	Nucleoside	Sponge	Cancer
	Vidarabine, Ara-A	Nucleoside	Sponge	Antiviral
	Ziconotide	Peptide	Cone snail	Pain
	Trabectedin (ET-743) (EU registered only)	Alkaloid	Tunicate	Cancer
Phase III	Eribulin Mesylate (E7389)	Macrolide	Sponge	Cancer
	Soblidotin (TZT 1027)	Peptide	Bacterium	Cancer
Phase II	DMXBA (GTS-21)	Alkaloid	Worm	Cognition, Schizophrenia
	Plinabulin (NPI-2358)	Diketopiperazine	Fungus	Cancer
	Plitidepsin	Depsipeptide	Tunicate	Cancer
	Elisidepsin	Depsipeptide	Mollusc	Cancer
	PM1004	Alkaloid	Nudibranch	Cancer
	Tasidotin, Synthadotin (ILX-651)	Peptide	Bacterium	Cancer
	Pseudopterosins	Diterpene glycoside	Soft coral	Wound healing
	Phase I	Bryostatin 1	Polyketide	Bryozoa*
Hemiasterline (E7974)		Tripeptide	Sponge	Cancer
Marizomib (Salinosporamide A; NPI-0052)		$\beta$ -lactone- $\gamma$ -lactam	Bacterium	Cancer

\*: Evidence of bacterial origin, see text.

Among the advantages of marine drug leads is a hypothesis that compounds of marine origin are very potent due to the inevitable dilution that occurs in this environment, and the structural diversity of the molecules is stunning (Grabowski *et al.*, 2008). An additional distinct advantage of

pursuing bacterial metabolites as drug leads is that, presumably, biosynthetic pathways of bacterial origin are more amenable to heterologous recombinant expression in for instance *E. coli*, which has the potential to eliminate the very real problem of a stable, cost-efficient supply of compound for tests and clinical trials (Molinski *et al.*, 2008). A recent success of biodiscovery within marine bacteria is the compound salinosporamide A. Salinosporamide A is a potent 20S proteasome inhibitor in clinical trials as anti-cancer agent (Feling *et al.*, 2003). It was discovered from a novel streptomycete and the development process included several valuable proof-of-concept milestones for marine drugs, for instance the implementation of a seawater based fermentation process in a cGMP setting (Fenical *et al.*, 2009).

### **2.0.1 THE MARINE ENVIRONMENT AS A SOURCE OF NOVEL MICROBIAL AND CHEMICAL DIVERSITY**

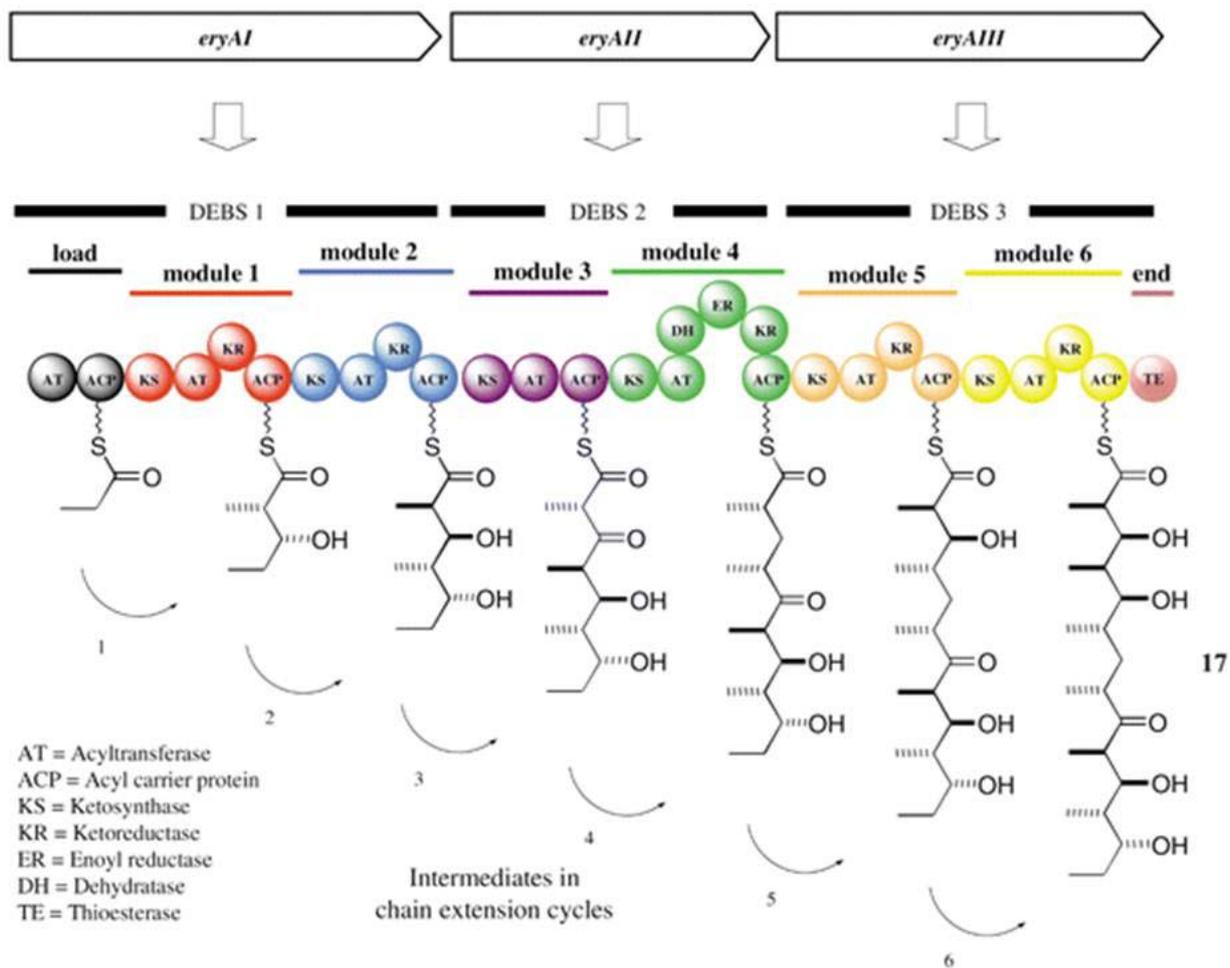
In the past, a common view was that the oceans were depleted in nutrients and presented harsh environmental parameters such as high salinity and a changing pH, which would not allow for microbial growth (MacLeod, 1965). This perception has changed, and today the marine environment is known to be teeming with microbial life considered a promising source of novel microbial and chemical diversity (DeLong, 2007). A high diversity of marine bacteria are often found in marine surface associated microbial communities (Holmström *et al.*, 2002; Webster *et al.*, 2001). Increasing evidence suggests that marine microbial communities can be highly specific to particular niches. A comparison of the surface communities of co-occurring marine eukaryotes revealed distinct bacterial communities when examined at the species level (Longford *et al.*, 2007), which is in agreement with later studies in which bacterial communities were found to be host-specific to several algae species (Lachnit *et al.*, 2009). The bacterial community associated with marine sponges consists of specialist bacteria specific to a certain sponge species, sponge associated bacteria that are found on several sponge species but not in seawater and generalist marine bacteria found both on sponges and in seawater (Taylor *et al.*, 2004). Even within one host individual, the composition of the epibiotic bacteria may be spatially distinct as reported for sponges (Meyer & Kuever, 2008; Thiel *et al.*, 2007) and algae (Staufenberger *et al.*, 2008). The niche specificity of bacterial communities is conceptually useful when trying to understand the vast diversity of marine bacteria and the implications on natural products discovery. Antagonistic

interactions among marine bacteria are frequently observed, and bacterially produced antibiotics are believed to be involved in microbial interactions and affect the structure of microbial communities (Long & Azam, 2001; Rypien *et al.*, 2010; Slattery *et al.*, 2001). In a recent study attempting to clarify interaction dynamics in soil microbial communities, a large number of pairwise interactions were observed among streptomycetes isolated from individual soil grains (Vetsigian *et al.*, 2011). Based on these interactions, the authors suggest a model in which the original microbial community is highly likely to evolve and adapt rapidly through for instance acquisition or adaptation of antibiotic producing genetic pathways, subsequently countered by the emergence of antibiotic resistance (Vetsigian *et al.*, 2011). It stands to reason that molecules involved in such interactions may evolve as microbial communities change, and thus microbial diversity and the assembly of diverse microbial communities may drive the evolution of chemical diversity. Hence, the understanding that many antibacterial compounds are involved in microbial interactions and shaping of microbial communities combined with the knowledge that microbial communities often are niche specific ties a link from microbial ecology to discovery of novel bioactive compounds (Imhoff *et al.*, 2011). Understanding the implications of microbial diversity and the ecology of antibiotics may be of great assistance in maximizing the output of biodiscovery efforts. Developments in analytical techniques such as MALDI-TOF-MS (Esquenazi *et al.*, 2008) have the potential to be of great assistance in research on the spatial location and identification of secondary metabolites within complex communities which has the potential to offer new and exciting insights at the interface of microbial ecology and natural products chemistry.

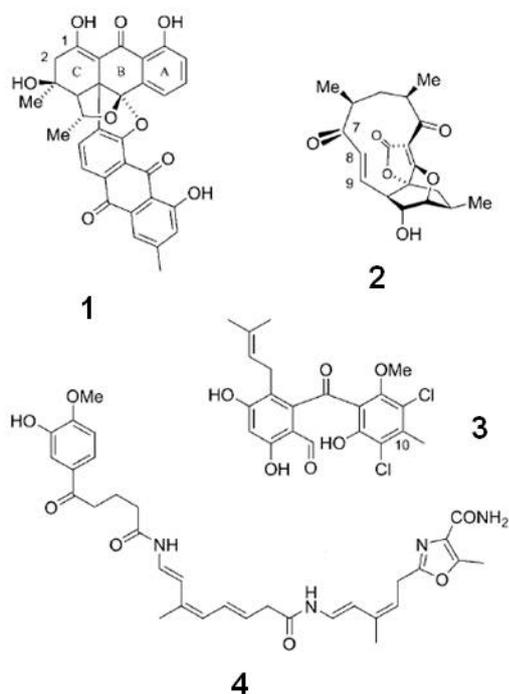
## **2.0.2 BIOSYNTHESIS OF BACTERIAL SECONDARY METABOLITES**

Bioactive natural products of bacterial origin cover an enormous structural diversity and include multiple compound classes, such as terpenes, polyketides and nonribosomal peptides. These compounds are synthesized by cellular enzymes which are often encoded by genes organized on the same locus in so called biosynthetic pathways. Two of the classes with a well understood biosynthetic pathway are polyketides (PKs) and nonribosomal peptides (NRPs), which are often isolated from marine microorganisms (Hughes & Fenical, 2010). The synthesis machineries for these two classes of compounds are conceptually alike. The active domains are contained within

one large protein in a modular structure where the synthesized molecule is transferred from one domain to the next as the catalytic reactions proceed (Staunton & Weissman, 2001). The canonic type I PK biosynthetic machinery depicted in figure 3. In PK synthesis, each module mediates one extension step of the PK chain, including any modification such as reduction of a ketone to a hydroxyl. Examples of antibacterial polyketetids of marine origin are shown in figure 4 below.

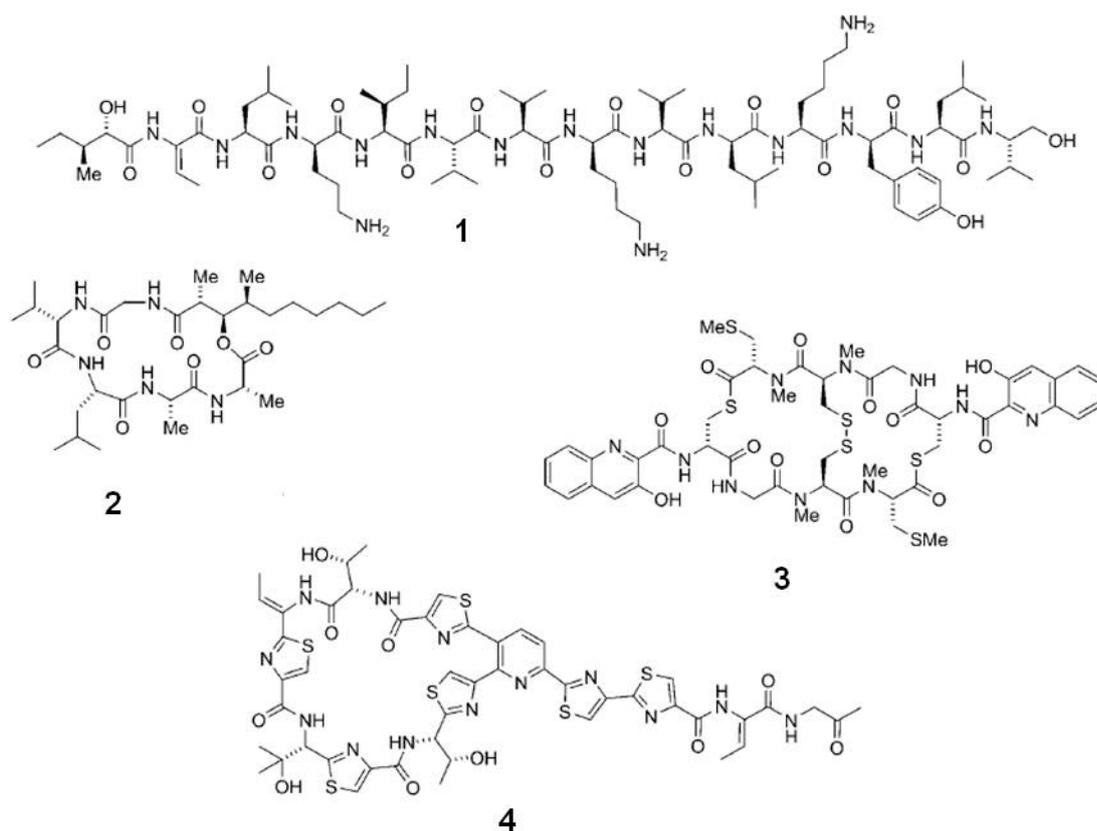


**Figure 3:** Modular organization of erythromycin polyketide biosynthesis, a PKS type I pathway. Each module (except the loading and end modules) contains the essential ketosynthase, acyltransferase and acyl carrier protein domains required for elongation of the polyketide chain, whereas additional domains alter the substitutions on the chain. Adapted from Staunton and Weissman (2001).



**Figure 4:** Antibacterial polyketides of marine origin. BE-43472B (**1**) isolated from a marine *Streptomyces*; abyssomycin C (**2**) isolated from the marine actinomycete *Verrucosispora* sp; pestalone (**3**) isolated from a marine fungus; ariakemicin A (**4**) isolated from a marine gliding bacterium of the phylum *Bacteroides*. Modified from Huges & Fenical (2010).

A key difference among the two pathways is the use of different building blocks; polyketide synthetases (PKSs) rely on acetate (activated to form malonyl-CoA) and propionate (activated to form methyl-malonyl-CoA) (Cortes *et al.*, 1990) whereas nonribosomal synthetases (NRPSs) utilize amino acids (Schwarzer *et al.*, 2003). Variations of these archetypical PKS molecules exist, for instance a PKS gene cluster with no intrinsic AT domain (Piel, 2002). Instead, this pathway relies on discrete AT genes to synthesize the end product pederin. Antibacterial NRPS molecules are produced by marine bacteria (figure 5), and the generalized pathway shares the modular layout with PKS pathways, however the domains encode different functionalities. Marine NRPs include violacein and indolmycin, isolated from *P. luteoviolacea* strains in this Ph.D. work, which can be recognized as NRP compounds based on their tryptophan building blocks. Hybrid pathways including both PKS and NRPS domains are known (Shen *et al.*, 2001). Such hybrid pathways further add to the potential structural diversity among natural products.



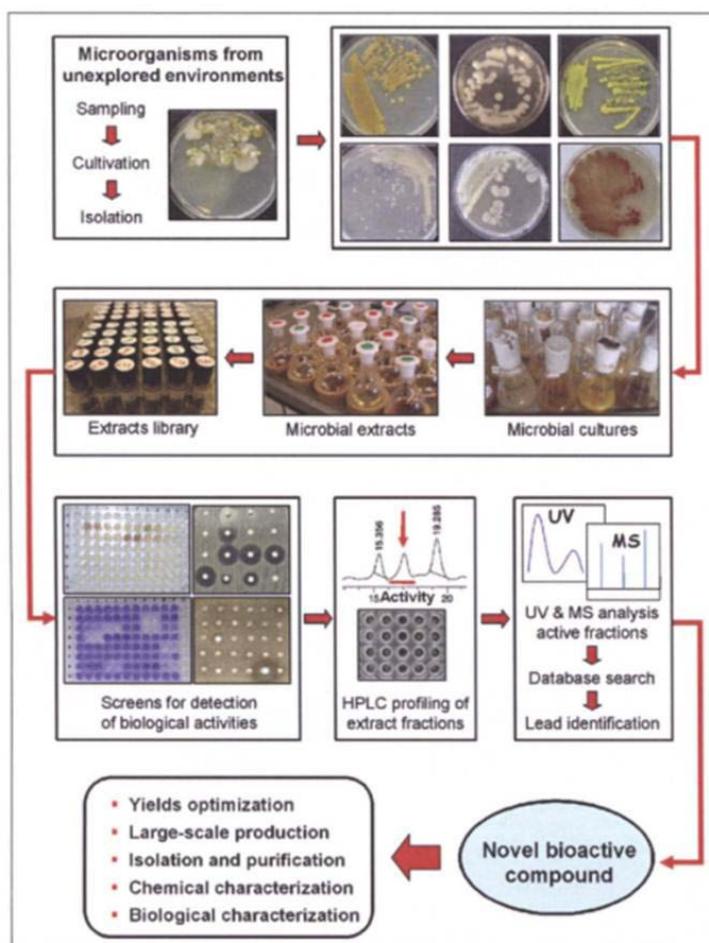
**Figure 5:** Antibacterial nonribosomal peptide-derived molecules of marine origin. Bogorol A (**1**) isolated from the marine *Bacillus laterosporus*; emericellamide A (**2**) isolated from a marine fungus; thiocoraline (**3**) isolated from a marine actinomycete; YM-266183 (**4**) isolated from a marine *Bacillus cereus*. Modified from Hughes & Fenical (2010).

The modular nature of these biosynthetic machineries has led to interest in so called combinatorial biosynthesis as reviewed by (Weissman & Leadlay, 2005), where the enzymes catalyzing the synthesis reactions are modified based on the desired outcome (Cortes *et al.*, 1995). For instance, adding enoyl reductase domain to a module can eliminate a functional group and change the steric configuration of the molecule, thus potentially altering the biological activity. This metabolic engineering is gradually becoming ‘plug-and-play’ through the availability of distinct building blocks (Menzella *et al.*, 2005). This approach opens for a virtually unlimited cache of structural scaffolds, and provides researchers with the ability to directly modify sites which are identified as important for pharmacological activity.

## 2.1 STRATEGIES FOR DISCOVERY OF ANTIBACTERIAL COMPOUNDS FROM MARINE BACTERIA

### 2.1.1 SCREENING PIPELINE FOR *PSEUDOALTEROMONAS* STRAINS IN THE GALATHEA 3 PROJECT

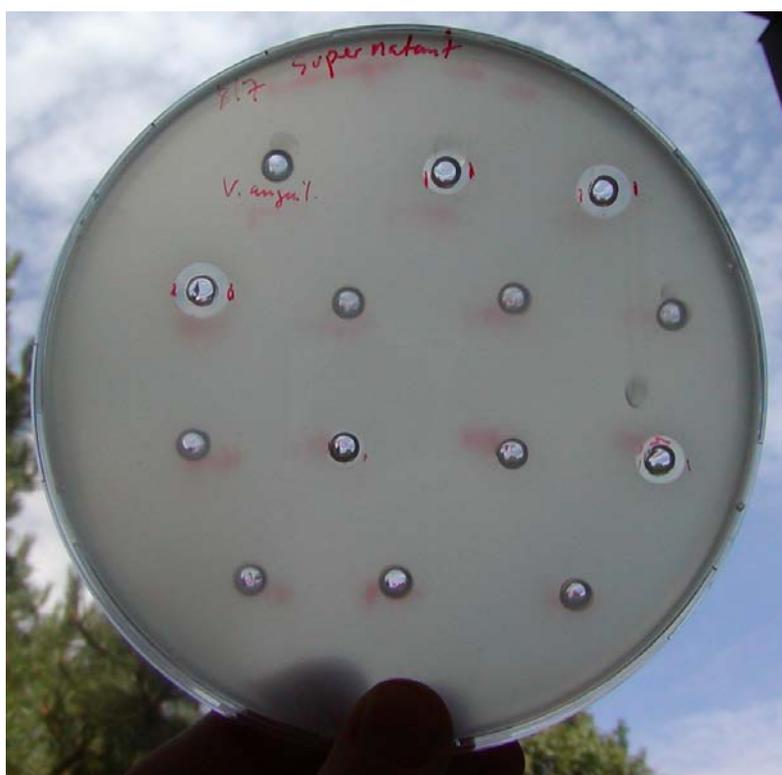
In this Ph.D. work, a screening pipeline was used to facilitate efficient screening for antibacterial compounds among the bacteria isolated during the research expedition Galathea 3. Bacterial strains were isolated from a range of diverse marine samples during the global expedition (Gram *et al.*, 2010) and pre-screened for antagonism towards *Vibrio anguillarum* 90-11-287 (Skov *et al.*, 1995), a potent fish pathogen. The bacterial strains were tentatively identified via BLAST queries of partial 16S rRNA gene sequences, and one of the dominant taxa was the genus *Pseudoalteromonas* which forms the topic of this thesis. The potentially antagonistic bacterial strains were screened for inhibitory activity following a principle similar to the outline in figure 6.



**Figure 6:** An overview of the principles in a screening strategy such as the one used in this Ph.D. work. Adapted from Molinari (2009).

The chemical analysis pipeline includes HPLC coupled with a detector, for instance an UV/VIS diode-array-detector. The HPLC is used to separate compounds in a given mixture by size, polarity or charge, resulting in a series of fractions which can be tested in the bioassay. Once a pure compound has been isolated, LC-MS can be used to obtain the accurate mass of the compound. The accurate mass is used to calculate the elemental composition and in combination with the formation of distinct adducts during LC-MS analysis, the compounds can often be dereplicated and identified with help from database searches (Nielsen *et al.*, 2011). However, to obtain the full structural information one must resort to nuclear magnetic resonance spectroscopy or x-ray crystallography.

The main bioassay used was a simple well diffusion agar assay (Hjelm *et al.*, 2004), where a target culture is cast into an agar substrate and test substances are added to wells cut in the agar (Figure 7).



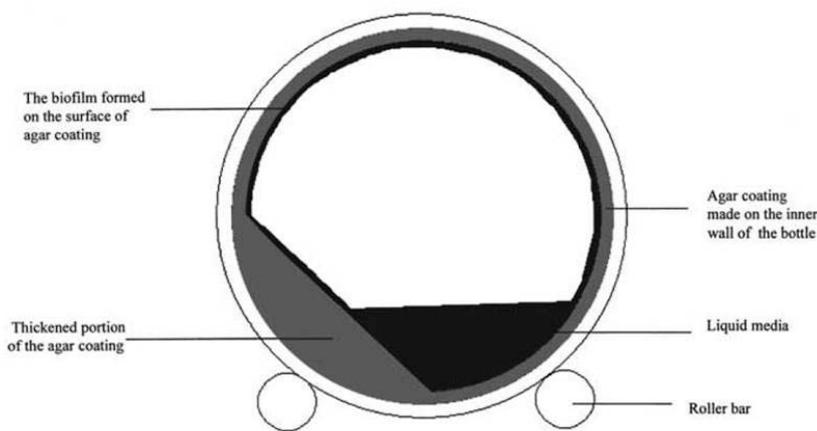
**Figure 7:** Well diffusion agar assay of sterile filtered *Pseudoalteromonas* culture supernatants. The inhibition of *Vibrio anguillarum* is seen as clearing zones of no bacterial growth, signifying the presence of an inhibitory compound in the tested supernatant.

This bioassay provides a very direct assessment of the antibacterial potential of a given sample, and repeated cycles of chemical fractionation followed by bioassays to verify activity is a proven method of obtaining relatively pure active compounds. This approach is often referred to as

bioassay guided fractionation. This principle can be used for the identification of practically any compound where a specific bioassay exists, however, as part of the work in this thesis shows (Vynne *et al.*, 2012c), complications such as different detection limits among bioassays and chemical detectors or the inability to confine the active compound to one single fraction may occur. Also, as subsequent paragraphs reveal, the genome of *P. luteoviolaceae* potentially points to a range of bioactive compounds that are not expressed under standard laboratory growth conditions.

### **2.1.2 CULTURABILITY AND THE INFLUENCE OF CULTURE CONDITIONS ON DISCOVERY OF SECONDARY METABOLITES FROM MARINE BACTERIA**

When determining if a bacterial strain produces for example antibiotics, growing the organism in pure cultures under controlled conditions is in most cases considered essential, and this approach has been greatly successful. It offers the immediate advantages of working with a pure culture growing under defined conditions for a high degree of reproducibility. Nevertheless, the fact that only a fraction of the cells in a given sample are culturable using standard laboratory approaches and growth substrates (an estimated 0.1 % for bacterioplankton (Gram *et al.*, 2010)) suggests that a reliance on these classic techniques may also limit the potential for discovery of novel antibiotics. To compensate for this, several authors have taken alternative approaches to cultivation. A simple modification to growth conditions in liquid cultures was developed by (Yan *et al.*, 2002) (figure 8). This presumably allows for increased biofilm formation and reduces oxygen limitations, and has the potential advantage of decreased exposure to autoinhibitory compounds. Culturing two marine *Bacillus* strains in this roller bottle setup induced production of two antimicrobial compounds which inhibited an MRSA test strain (Yan *et al.*, 2002). Similarly, growth in biofilms may induce or enhance production of antibiotics as demonstrated for *Phaeobacter* 27-4 (Bruhn *et al.*, 2007). This illustrates how important the physical conditions can be to antibiotic production.



**Figure 8:** Agar coated roller bottle design. The bottle rotates at 1 rpm, and bacterial biofilms form on the agar coated interior walls. The thickened portion of the agar allows for aeration of the liquid substrate. Adapted from Yan *et al.* (2002).

Laboratory growth substrates are often based on either a complex mix of carbohydrates and nitrogen designed to support fast bacterial growth, for instance yeast extract or peptone, or defined minimal substrates designed for selective enrichment of bacterial cultures or to support fastidious organisms. In the case of marine bacteria, neither substrate is likely to reflect the natural niche very and thus may not induce the production of key secondary metabolites. A recent study elegantly demonstrated how growth substrates may impact secondary metabolite production: A *Vibrio corallilyticus* strain shut down production of all secondary metabolites but the antibiotic andrimid when cultured on chitin or live chitinous *Artemia* instead of marine broth 2216 (Wietz *et al.*, 2011). This validates the use of growth substrates mimicking the natural nutrient regime, and is an excellent illustration of how microbial ecology and biodiscovery may go hand in hand. Preliminary results obtained during this Ph.D. work of *P. luteoviolacea* cultures grown on alga extracts suggests this strategy may indeed change the metabolic profile of the bacterium, and cultures grown in the complex growth substrate heart infusion broth supplemented with 2% sodium chloride caused a heavy production of extracellular polymers in the strain *P. luteoviolacea* S4054 (unpublished data).

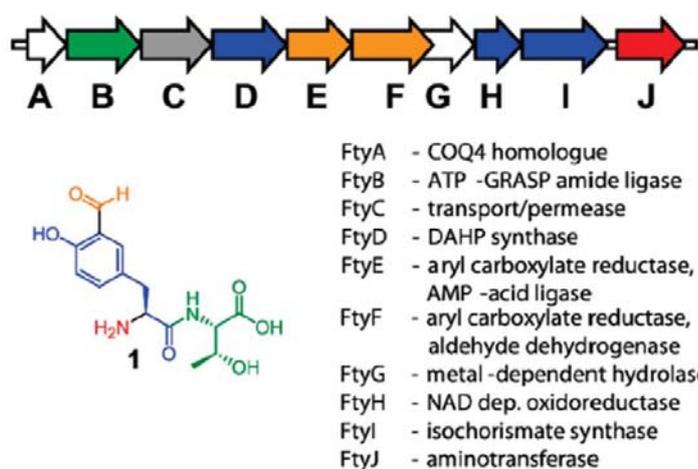
Elicitation of antibiotic production may be achieved by co-culturing the producer organism with other microorganisms in an effort to simulate environmental parameters from the natural environment, such as signaling and stress factors. One of the earliest studies involving marine bacteria demonstrated how marine bacteria exposed to cells of *S. aureus* or *Pseudomonas aeruginosa* responded with increased antibacterial activity compared to non-exposed controls

(Mearns-Spragg *et al.*, 1998). Similar results were obtained for marine sponge-associated bacteria (Kanagasabhapathy & Nagata, 2008) and in a marine streptomycete (Slattery *et al.*, 2001). Three of four tested tropical marine epibiotic bacteria showed antifungal activity when exposed to live cells of the fungus for 24 h, whereas pure cultures of the epibiotic bacteria were not antifungal (Dusane *et al.*, 2011). This is a logical extension of the hypothesis that antibiotics have ecological significance, and conceivably are produced as chemical weapons in response to being challenged by foreign competitor cells. However, in some cases the problem itself is accessing the secondary metabolism of a so called unculturable microorganism. The use of high-throughput microtiter plate techniques, dilution methods and a growth substrate containing natural levels of nutrients led to the culturing of 14% of the cells in a marine water sample (Connon & Giovannoni, 2002), compared to the ca. 0.1% normally achieved (Gram *et al.*, 2010). Among the cultures were several novel Proteobacteria clades. One of these clades was related to the SAR11 lineage, which is considered ubiquitous in the ocean surface layer and may contribute up to 50% of the total bacterioplankton cell numbers (Morris *et al.*, 2002). SAR11 does not grow at standard laboratory techniques, but the use of the high-throughput, low nutrient technique allowed the successful culturing of SAR11 bacterial strains (Rappe *et al.*, 2002) and subsequent improvements to the technique have allowed the culturing of more diverse novel SAR11 strains (Stingl *et al.*, 2007). Other approaches include the use of various growth factors (D'Onofrio *et al.*, 2010; Nichols *et al.*, 2008), diffusion chambers (Bollmann *et al.*, 2007) and membrane systems (Ferrari *et al.*, 2008). Together, these innovative approaches to the fundamental microbiological task of culturing bacteria have opened new possibilities for culturing parts of the previously rarely isolated or unculturable majority of bacteria (Sogin *et al.*, 2006), which allows access to practically unlimited diversity.

### **2.1.3 GENOMICS AND METAGENOMICS AS TOOLS IN BIODISCOVERY: A MARINE PERSPECTIVE**

As the first bacterial genomes of prolific secondary metabolite producers were completed, it was observed that they contained gene clusters with homology to known biosynthetic pathways, but to which no known secondary metabolite could be associated. Although advanced culture techniques may activate some of these silent clusters one faces a daunting task if attempting to

elicit every biosynthetic pathway without further knowledge as for instance a genome sequence. With sequencing technologies rapidly advancing and sequencing costs dropping equally fast, genome and metagenome sequencing has become accessible to most labs in terms of both costs and the required expertise. Excellent software exists to aid in handling the large amounts of data generated by these approaches, and the modular nature of PKS and NRPS gene clusters makes *in silico* prediction of homologue loci an exciting opportunity. In fact, genome sequencing and analysis has already proven itself in discovery of previously non-described compounds from otherwise thoroughly studied bacteria. Prediction of novel secondary metabolites from such silent gene clusters are known from *Streptomyces coelicolor* (Challis & Ravel, 2000), where a silent NRPS pathway was shown to produce the novel iron chelator coelichelin under iron-limited growth conditions (Lautru *et al.*, 2005). One of the first reports on secondary metabolites from thermophilic bacteria was based on genome analysis and identification of an orphan NRPS gene cluster in the thermophilic actinomycete *Thermobifida fusca*. The biosynthetic pathway showed unusual features including non-linear peptide assembly, and the end product was a siderophore molecule possessing a distinct novel iron chelation domain (Dimise *et al.*, 2008). In the genome of the marine bacterium *Pseudoalteromonas tunicata* genome, a biosynthetic gene cluster was predicted based on the presence of an ATP-grasp domain (Blasiak & Clardy, 2009). The putative biosynthetic gene cluster, shown in figure 9, was then PCR amplified and cloned into a vector under control of an inducible promoter, and transformed into an *E. coli* host. Induction of the promoter resulted in the production and subsequent identification of two 3-formyl-tyrosine metabolites which were not previously identified in the otherwise well-studied *P. tunicata* strain D2 – possibly due to their lack of antibacterial activity.



**Figure 9:** Predicted biosynthetic gene cluster from the *P. tunicata* D2 genome. 1; structure of 3-formyl-L-tyrosine-L-threonine dipeptide, one of the discovered metabolites. Color codes indicate the proposed biosynthetic pathway. Adapted from Blasiak and Clardy (2010).

In this Ph.D. work, the genome sequence of *P. luteoviolacea* S4054 revealed between 7 (manually curated annotation of PKS and NRPS containing gene clusters in IMG/ER (Vynne & Gram, 2012)) and 14 (antiSMASH prediction (Medema *et al.*, 2011)) potential biosynthetic pathways, one of which was the violacein pathway. The violacein pathway is known (Antônio & Creczynski-Pasa, 2004; August *et al.*, 2000; Hoshino, 2011) and can be identified through homology searches, but none of the remaining potential biosynthetic pathways were homologous to pathways encoding known secondary metabolites. The antibiotics indolmycin and pentabromopseudilin were identified in *P. luteoviolacea* S4054 during this Ph.D. work, and their biosynthetic pathways are likely among the predicted gene clusters. However, since the genes involved in the biosynthesis are unknown, no pathway could be directly assigned to these compounds. The presence of up to 14 novel biosynthetic pathways opens the possibility that *P. luteoviolacea* S4054 may produce up to 11 as yet unknown secondary metabolites, highlighting the power of genomic analysis and the potential for genome assisted biodiscovery in marine *Pseudoalteromonas*.

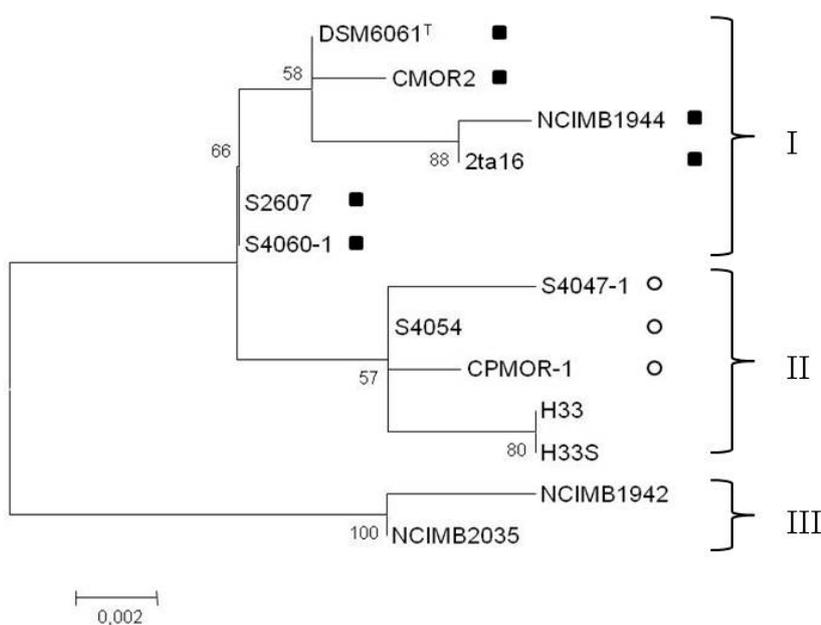
Metagenomics have shown a potential as a biodiscovery related technique, as evidenced by the Global Ocean Sampling expedition, which based on the DNA sequence data obtained predicted a multitude of unknown proteins and protein families (Yooseph *et al.*, 2007). However, a general limitation of homology based studies is the reduced chance of finding something very different from what is already known, as genetic homology does imply a structural or conceptual similarity among the end products. Hence, *in silico* genomic and metagenomic studies offer a fantastic supplement to classical culture-based approaches but cannot be relied upon as the sole means of assessing the secondary metabolite diversity of a single strain or microbial community. A more

direct approach is using metagenomic DNA directly to create libraries of clones containing large inserts of metagenomic DNA and perform functional screens for expression of e.g. antibacterial activity. In a pioneering study on functional screening of uncultured marine symbionts, a functional screen of shotgun cloned 'metagenomic' DNA (mainly *Prochloron* sp.) led to the cloning and characterization of the biosynthetic pathway of the cytotoxic compound patellamide D DOI: 10.1002/cbic.200500210. A fosmid library was used to screen metagenomic DNA from microbial communities associated with a sponge (*Cymbastela concentrica*) and an alga (*Ulva australis*) for novel antibacterials, which resulted in the identification of three novel antibacterial proteins (Yung *et al.*, 2011).

## 2.2 BACTERIAL TAXONOMY IN BIODISCOVERY

Proper identification and taxonomical assignment is crucial to scientific communication, also within natural product discovery (Jensen, 2010). The production of secondary metabolites is often considered to be strain specific (Waksman & Schatz, 1943), in part due to the involvement of biosynthetic gene clusters in lateral gene transfer (LGT) (Egan *et al.*, 2001b). This would seem to exclude bacterial taxonomy as a useful tool for dereplicating bacterial strains in a biodiscovery setting, however screening of a taxonomically dereplicated collection of actinomycetes led to a high number of discovered compounds compared to the strain throughput (Goodfellow & Fiedler, 2010). Studies of the secondary metabolites produced by *Salinispora* species showed that a core set of metabolites are restricted to specific species (Jensen *et al.*, 2007; Penn *et al.*, 2009) and among species of the *Roseobacter* clade, production of a brown pigment and the antibacterial compound tropodithietic acid was shown to be related to growth conditions in a species-specific manner (Porsby *et al.*, 2008). Hence, species specific chemoprofiles can exist. This is valid for some filamentous fungi as well, and has been used for guided discovery of secondary metabolites (Frisvad *et al.*, 2008). Since the available resources often limit the amount of strains that can be included, not screening strains with identical or overlapping secondary metabolite profiles would be beneficial in biodiscovery efforts. In this Ph.D. work, a comparison between a statistical clustering based on secondary metabolites and 16S rRNA gene sequence phylogeny was made to reveal the extent, if any, of species specific secondary metabolite production in the

*Pseudoalteromonas* genus (Vynne *et al.*, 2011). Some strains, such as *P. ruthenica* or the *P. aurantia* – *P. citrea* complex were found to cluster together as expected based on 16S rRNA sequence identity. However, some species, e.g. *P. luteoviolacea*, were split in distinct clusters by chemoprofiling and strains of *P. rubra* were very diverse from a secondary metabolite point of view. This study showed that 16S rRNA gene sequences are insufficient for reliable dereplication of secondary metabolite profiles in *Pseudoalteromonas* strains. However, in a follow up study it was demonstrated that a more detailed phylogenetic analysis of GyrB and RecA sequences were capable of resolving phylogenetic groups matching the observed distribution of bioactive secondary metabolites (figure 10) (Vynne *et al.*, 2012b). In addition, the strains that produced the antibiotic indolmycin were exclusively isolated from algae, suggesting an ecology aspect is involved in production of this compound. The fixation of secondary metabolites within phylogenetic clusters is a strong indication that the biosynthetic gene clusters are under selective pressure and thus play an important functional role (Jain *et al.*, 1999), which further supports the importance of antagonistic compounds in microbial ecology. Furthermore, the detailed phylogeny could be used as a tool for dereplication of bacterial strains in biodiscovery.



**Figure 10:** Phylotype specific production of bioactive compounds within *P. luteoviolacea*. The phylogenetic tree was based on concatenated GyrB and RecA sequences. Closed boxes: pentabromopseudilin producer, open circles: indolmycin producer. All strains produced violacein. Adapted from Vynne *et al.* (2011).

## 2.3 CONCLUSIONS FROM CHAPTER 2

Marine bacteria are a proverbial ‘treasure trove’ of bioactive natural compounds, many of which are likely to possess interesting pharmacological activities. Advances in microbial ecology may be of great use within biodiscovery programs, as these bioactive compounds are highly likely to be involved in microbial ecology. For instance, sampling strategies could benefit from targeting specific niches if a certain biological activity was desired, or could simply aim at obtaining samples from as diverse microbial communities as possible. Once the biological material is collected, a dereplication of the bacterial strains may be carried out based on phylogeny and knowledge of microbial ecology to reduce the inclusion of overlapping secondary metabolomes. The biosynthetic potential of a *Pseudoalteromonas luteoviolacea* strain was assessed using bioinformatic approaches, and was found to contain up to 14 pathways involved in secondary metabolism. Accessing the biosynthetic potential via both culture dependent and culture independent techniques is likely to produce the best results, and modulation of growth conditions play an important role in this process.

### 3.0 THE GENUS *PSEUDOALTEROMONAS* – ECOLOGY AND BIOACTIVITY

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The genus *Pseudoalteromonas* consists of obligate marine bacteria found ubiquitously in the marine environment. They are Gram-negative non-sporeforming straight or curved rods belonging to the  $\gamma$ -*Proteobacteria*. The cells are motile by one or more polar flagella (Romanenko *et al.*, 2003) with some species reported to have lateral flagella in addition to polar. They are oxidase positive, vary in catalase reaction and are non-fermenting with a genomic DNA GC content of 37-50 mol% and several species are intensely pigmented (figure 11) (Gauthier *et al.*, 1995). Many *Pseudoalteromonas* show bioactivity such as antagonism towards other bacteria, hydrolytic enzymatic activities or anti-fouling activity. Pseudoalteromonads are considered obligate marine, but will grow on a range of nutrient regimes if marine salts are present. Some species, such as *P. ruthenica* can grow on NaCl as the sole salt source (Vynne, unpublished). All species grow at 20°C, with growth ranges within 4°C to 40°C. The bacteria are readily cultured on e.g. Marine Agar 2216 (Difco, USA) using routine culture techniques.



**Figure 11:** *Pseudoalteromonas* strains streaked on Marine Agar 2216.

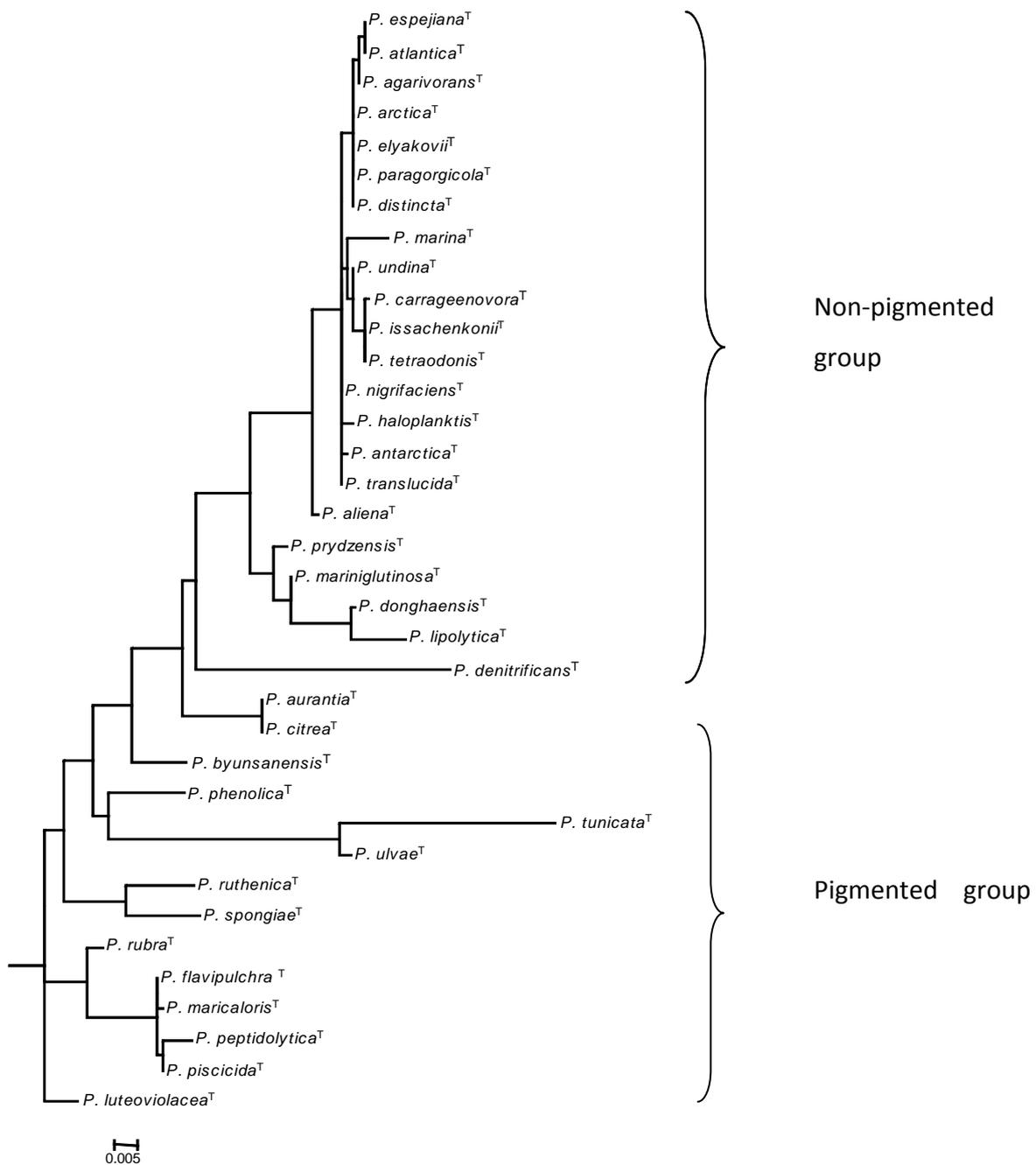
Non-pigmented: *P. agarivorans*,  
yellow: *P. flavipulchra*,  
dark purple: *P. luteoviolacea*  
red: *P. rubra*

The first descriptions of *Pseudoalteromonas* species were published in the 1940s and 1950s when the isolates were assigned to the *Flavobacterium*, *Pseudomonas* or *Vibrio* genera (Bein, 1954; Berland *et al.*, 1969; ZoBell & Upham, 1944). In 1972, the genus *Alteromonas* was created to harbor marine aerobic bacteria with GC-mol% of 42.8 to 48 and a polar flagellum (Baumann *et al.*, 1972), however with the advent of 16S rRNA gene sequence analysis it was clear that the genus

type strain *Alteromonas macleodii* formed a monophyletic cluster away from the remaining species in the genus (Gauthier *et al.*, 1995). To account for this discovery, the genus *Pseudoalteromonas* was created with *Pseudoalteromonas haloplanktis* as genus type strain and containing a total of 12 species. The genus now encompasses 36 species (Euzéby, 1997) including several that were recently described (Oh & Park, 2011; Xu *et al.*, 2010).

### **3.1 PSEUDOALTEROMONAS PHYLOGENY AND TAXONOMY**

Understanding and navigating the taxonomy of *Pseudoalteromonas* species requires some attention to detail, as many species share a very high (>99%) 16S rRNA gene sequence identity. This can complicate the designation of species names to novel isolates, in particular since phenotypic traits will also often be similar and it is not practical to do DNA-DNA hybridization for each interesting novel strain. Thus, the reader is encouraged to study the phylogenetic relations among the *Pseudoalteromonas* type strains presented in figure 12, and keep in mind that there is likely a degree of overlap in what different authors identify as e.g. *P. aurantia* or *P. citrea*. The importance of proper species designation should not be understated. Accurate identification of a novel isolate is key in order to communicate science and allow other scientists to perform satisfactory literature searches as stressed in a recent review on marine natural products (Blunt *et al.*, 2009). The use of 16S rRNA gene sequences to resolve bacterial taxonomy to the species level is widely accepted, cheap and fast to use. A 97% sequence identity is the accepted threshold for species delineation (Stackebrandt *et al.*, 2002), however above 97% sequence identity does not confirm two strains as one species (Stackebrandt & Goebel, 1994). The gold standard remains DNA-DNA hybridization among species type strains where less than 70% DNA similarity is required to separate two strains into distinct species (Wayne *et al.*, 1987), although the use of whole-genome sequence data may replace this method (Konstantinidis & Tiedje, 2005). The use of DNA-DNA hybridization, biochemical data and phylogenetic analysis of 16S rRNA genes is termed the polyphasic approach (Vandamme *et al.*, 1996).



**Figure 12:** Phylogenetic analysis of 16S rRNA sequences of *Pseudoalteromonas* type strains. Sequences were retrieved from GenBank, aligned in MEGA5 (Tamura *et al.*, 2011) and a phylogenetic tree inferred by maximum likelihood analysis. Scale bar: 0.005 nucleotide substitutions per site.

In the course of this Ph.D. study, strain S3431 was identified as a potentially novel *Pseudoalteromonas* species. The strain was originally isolated from a polychaete near Canal Concepcion in Chile and immediately caught our attention due to its deeply black pigment when cultured on marine agar 2216. Initial results of the S3431 16S rRNA gene sequence analysis showed that it had below 97% similarity to any described *Pseudoalteromonas* type strain. Further analyses of phenotypic traits and chemotaxonomic markers were conducted, and DNA-DNA hybridization was initiated. A later revision of the 16S rRNA sequence revealed sequencing errors and, after careful re-sequencing, the strain was found to have a 16S rRNA sequence identity of 99% to a number of non-pigmented *Pseudoalteromonas* type strains. However, it was decided that the preliminary results combined with the interesting pigmented phenotype was sufficient to warrant further investigations. DNA-DNA hybridizations (table 2), physiological data and biochemical tests revealed that S3431 represents a novel species given the name *Pseudoalteromonas galathea* (Vynne *et al.*, 2012a).

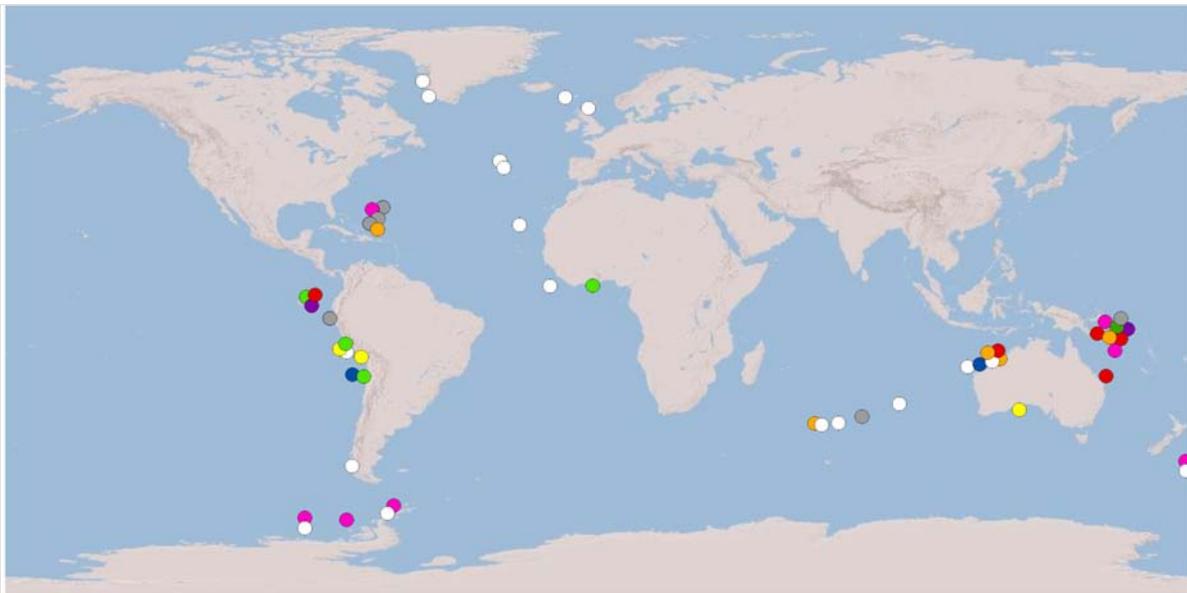
**Table 2:** Per cent DNA-DNA similarity in 2X SSC at 66°C (Vynne *et al.*, 2012a). All related type strains have less than 70% DNA-DNA similarity to S3431, which is the accepted threshold for species delineation (Wayne *et al.*, 1987).

<i>Pseudoalteromonas</i> species	Per cent DNA:DNA similarity to strain S3431
<i>P. agarivorans</i> DSM 14585 <sup>T</sup>	28.2
<i>P. aliena</i> LMG 22059 <sup>T</sup>	44.5
<i>P. atlantica</i> NCOMB 301 <sup>T</sup>	26.9
<i>P. carrageenovora</i> DSM 6820 <sup>T</sup>	14.8
<i>P. distincta</i> CIP 105340 <sup>T</sup>	44.5
<i>P. elyakovii</i> LMG 14908 <sup>T</sup>	44.2
<i>P. espejiana</i> DSM 9414 <sup>T</sup>	48.0
<i>P. issachenkonii</i> LMG19697 <sup>T</sup>	33.6
<i>P. nigrifaciens</i> LMG 2227 <sup>T</sup>	15.4
<i>P. paragorgicola</i> LMG 19696 <sup>T</sup>	58.4
<i>P. tetraodonis</i> DSM 9166 <sup>T</sup>	11.3
<i>P. undina</i> LMG 2880 <sup>T</sup>	13.8

Unfortunately, the antibacterial effect that the strain was originally isolated for turned out to not be reproducible upon frozen storage and repeated culture in the lab. This is not uncommon, and may be due to several factors such as a need for specific nutrients or a lack of interaction with for

instance co-cultured microorganisms. As described above, using culture conditions closer to the natural environment (C-source, associate microbiota) may re-evolve the production of antibacterial activity.

Figure 12 above reveals a characteristic feature of the genus, namely the phylogenetic division into two groups when 16S rRNA gene sequences of the type strains are analysed (Gauthier *et al.*, 1995; Ivanova *et al.*, 2004). One group predominantly consists of non-pigmented bacteria with little antibacterial activity while the majority of the other group is pigmented bacteria typically isolated from marine biotic surfaces. This latter group often produce compounds involved in microbial antagonism (Mikhailov *et al.*, 2002) and correlation between pigmentation and anti-fouling activity was reported for *P. tunicata* and *Pseudoalteromonas* sf57 (Huang *et al.*, 2011) where anti-fouling activity was lost in non-pigmented mutants. It is important to note that bioactivity is not exclusively linked to pigmentation as recently shown by Bernbom *et al.* (2011). A more comprehensive review is presented in the paragraph on bioactivity in *Pseudoalteromonas*.



**Figure 13:** Isolation sites of 101 *Pseudoalteromonas* strains with each color code representing one of the 8 clusters indicated in Table 1 in Vynne *et al.* (2011)

The present work investigated 101 novel environmental *Pseudoalteromonas* isolated globally from a range of marine sources (figure 13), and whilst all isolates in initial screenings displayed some

degree of antibacterial activity, reproducible inhibition of *Vibrio anguillarum* occurred significantly more frequent among pigmented strains (table 3). Analysis of 16S rRNA gene sequences supported the phylogenetic grouping of pigmented and non-pigmented strains respectively (Vynne *et al.*, 2011).

**Table 3:** Identity, antibacterial activity and pigmentation of *Pseudoalteromonas* strains from a global collection. Modified from Vynne *et al.* (2011)

16S rRNA cluster	No of strains		Related type strain	No of strains from		No of strains inhibiting <i>Vibrio</i>		<i>Vibrio</i> inhibition by EtAc extract
	pigmented	non-pigmented		Surface	Water	pigmented	non-pigmented	
I	1	37	<i>P.agarivorans</i>	16	22	0	0	0
II	5	0	<i>P.aurantia</i>	2	3	5	0	0
III	0	9	<i>P.prydzensis</i>	8	1	0	0	0
IV	3	3	<i>P.phenolica</i>	3	3	3	2	0
V	4	0	<i>P.luteoviolacea</i>	4	0	4	0	4
VI	9	0	<i>P.rubra</i>	9	0	9	0	0
VII	13	0	<i>P.flavipulchra</i>	13	0	13	0	0
VIII	15	0	<i>P.ruthenica</i>	14	1	15	0	15
S1712	0	1	<i>P.mariniglutinosa</i>	0	1	0	0	0
S3655	1	0	<i>P.spongiae</i>	1	0	0	0	0
Total	51	50		70	31	49	2	19

### 3.2 ECOLOGY OF PSEUDOALTEROMONAS

*Pseudoalteromonas* bacterial cells constitute 0.5-6% of the oceanic bacterioplankton (Wietz *et al.*, 2010) and 0.01%-2.1% of surface associated bacteria on a selection of marine surfaces (Skovhus *et al.*, 2004; Skovhus *et al.*, 2007). The strains included in this Ph.D. study originated from the open water column, from algae and seaweeds, fish skin or copepods as well as rocks and sediment, illustrating the wide range of environments to which *Pseudoalteromonas* species have adapted and successfully colonized.

The antagonistic *Pseudoalteromonas* species are frequently associated with marine microbial surface communities on biotic or abiotic surfaces (Gram *et al.*, 2010; Holmström & Kjelleberg, 1999). From an ecology point of view this makes sense, since a bacterium adapted to planktonic life will rarely want to find itself in a situation where production of antagonistic compounds would provide a competitive advantage. Intuitively one can imagine polar antagonistic compounds diffusing freely away resulting in concentrations much too low to exert their antagonistic effects, thus incurring a fitness (production) cost without any gains. Environmental biofilms consists of mixed microbial species and competition for nutrients and space is high (Egan *et al.*, 2008). Several strategies to overcome these challenges exist, e.g. production of antagonist compounds, production of EPS and modulation of bacterial and/or host signaling (Hibbing *et al.*, 2009). The high density of microbial cells in surface biofilms may also facilitate an increased rate of exchange of genetic material, allowing for exchange of e.g. biosynthetic pathways. Additionally the biofilm complex may physically limit the rate of diffusion (Stewart, 2003), making it easier for antagonistic species to maintain the local concentration of the bioactive compound.

Many *Pseudoalteromonas* species produce enzymes which degrade complex polysaccharides such as xylan, chitin or cellulose. In planktonic *Pseudoalteromonas* strains these enzymes likely allow rapid breakdown of particulate organic matter (POM) to support bacterial growth and survival, while surface associated *Pseudoalteromonas* could rely on such enzymes to break down host cell wall polysaccharides for nutrition. Cellulose induces assembly of type IV-like pili in *P. tunicata* which in turn promotes attachment to the algae *Ulva lactuca*, a known host of *P. tunicata* (Dalisay *et al.*, 2006) and thus could play a central role in initiating biofilm formation. It also suggests that *P. tunicata* may selectively colonize and feed on surfaces with specific available polysaccharides, providing a model for niche colonization within marine bacteria. It is hypothesized that some *Pseudoalteromonas* species may also colonize and interact with higher eukaryotes to the benefit of the host organism. In this scenario, the *Pseudoalteromonas* produce antagonistic compounds to e.g. protect the host from excessive fouling (Boyd *et al.*, 1999; Dobretsov & Qian, 2002; Holmström *et al.*, 2002) and may in turn acquire nutrients either by scavenging directly from the host via enzymatic degradation or from the increased concentration of organic matter found on any

marine surface (Wahl, 1989). These are examples of how *Pseudoalteromonas* species have adapted to life on marine surfaces. In the present study, the strains with antagonistic effects towards *Vibrio anguillarum* and *Staphylococcus aureus* were significantly more likely to originate from a surface (Vynne *et al.*, 2011). This is consistent with bacterial antagonism as a selective advantage in the competitive environment of marine surfaces. However it was previously reported that the overall abundance of Pseudoalteromonads was lower in surface associated communities than in bacterioplankton (Skovhus *et al.*, 2007). This is likely a reflection of the multiple strategies that exist for microbial competition in surface communities.

### **3.2.1 PSEUDOALTEROMONAS SPECIES INTERACT WITH HIGHER EUKARYOTES**

It is known that some marine host-microbe interactions are highly specific, to the point of a symbiosis. *Pseudoalteromonas* species are known to be associated with higher eukaryotes (Holmström & Kjelleberg, 1999), and the suite of bioactive compounds produced by some species may relate to such interactions. The macroalga *M. oxyspermum* will not develop its normal morphology unless it is colonized by certain marine bacteria (Tatewaki *et al.*, 1983). Similarly, larvae of many bivalve species are more likely to settle where bacteria that secrete a chemical cue have already established (Hadfield & Paul, 2001). Indeed, biofilms of some *Pseudoalteromonas* species induce settlement of eukaryote larvae to actively promote macrofouling (Huggett *et al.*, 2006; Tebben *et al.*, 2011), while other species deter settlement (Holmström *et al.*, 1992). Crustose coralline algae (CCA) are important in marine ecology as several organisms including sea urchin feed on these calcifying algae, and studies of CCA epiphytic bacteria provide excellent insight into the complexity of *Pseudoalteromonas* - eukaryote interactions. Huggett *et al.* (2006) reported that *Pseudoalteromonas* strains were among the bioactive bacteria isolated from a crustose coralline alga, and that some greatly induced settlement of larvae from the sea urchin *Heliocidaris erythrogramma* while other Pseudoalteromonads were poor inducers of larval settlement. Upon closer inspection, a BLAST search reveals that strains related to *P. luteoviolacea* were found in both the highly and poorly inducing group. It is tempting to speculate that some of these bacteria produce compounds with cytotoxic activity towards sea urchin to protect the CCA host although no direct evidence of this exists. Furthermore, it would be of interest to perform a detailed

phylogenetic study on these *P. luteoviolacea* strains, as this Ph.D. study has revealed a correlation between phylogeny and production of bioactive compounds (Vynne *et al.*, 2012b) and extending this approach could reveal any phylogenetic patterns in larval interactions which may be related to production of small-molecule compounds (Tebben *et al.*, 2011). Furthermore, a bachelor student in our group recently demonstrated that some strains of *P. luteoviolacea* are highly toxic to live *Artemia nauplii* whilst others have no adverse effect. This division reflected the antibiotic profile of the strains, since two strains producing pentabromopseudilin killed *Artemia* whereas two strains producing indolmycin had no effect (Anna Neu, unpublished data).

The *Pseudoalteromonas* eukaryote interactions could represent a strategy to attract potential host organisms and repel undesired settlement of macroscopic species, providing an indication of a microbe-host relationship with some symbiotic characteristics.

### **3.3 PSEUDOALTEROMONADS AS PROBIOTICS IN AQUACULTURE**

The increase in fish rearing for human consumption has been dramatic and comes at a cost to the environment in part due to the accompanying use of antibiotics (Cabello, 2006). Probiotics have been defined by FAO/WHO as "live microorganisms which when administered in adequate amounts, confer a health benefit on the host." (FAO and WHO, 2001) Adding live antagonistic marine bacteria to an aquaculture system has reduced mortality in certain models and could reduce the need for antibiotics (Gatesoupe, 1997; Moriarty, 1998; Planas *et al.*, 2006; Rengpipat *et al.*, 1998). This provides a basic 'proof-of-concept' that antagonistic bacteria can act as probiotics. Based on their bacterial antagonist properties, *Pseudoalteromonas* strains are candidates for application as probiotics. Longeon *et al.* describes the probiotic effect of *Pseudoalteromonas* sp. X153 which produces an antibacterial protein of 87 kDa (Longeon *et al.*, 2004). Rearing of scallop in co-culture with *Pseudoalteromonas* sp. X153 significantly reduced scallop mortality but also, due to reasons unknown, slightly reduced the scallop growth rate. Interestingly, 16S rRNA gene sequence analysis showed a 99.9% identity to *P. piscicida*, a known fish pathogen. A co-culture of three commensal strains, one of them identified via 16S rRNA gene sequence similarity as *P. elyakovii*, of larvae of Atlantic halibut was shown to improve survival of the larvae when grazing on

bacteria inoculated prey (Bjornsdottir *et al.*, 2010). Probiotic functions are not restricted to promoting animal life, as illustrated by the ability of a marine bacterium to enhance the growth rate of a range of plant species due to production of an enzyme with catalase activity (Dimitrieva *et al.*, 2006). The bacterium was assigned to the Pseudoalteromonads based on biochemical and physiological tests, and identified by the authors as *Pseudoalteromonas porphyrae* based on 16S rRNA gene sequence similarity. However, the bacterium should likely be referred to as a strain of *P. atlantica* or *P. elyakovii* (both >97% 16S rRNA gene sequence similarity) as *P. porphyrae* is currently not an accepted species designation (Euzéby, 1997). These studies illustrate the potential of Pseudoalteromonads to function as probiotics within diverse aquaculture applications, and provide a glimpse into the possible roles played by assorted *Pseudoalteromonas* strains in the marine environment. In this thesis work, the fish pathogenic bacterium *Vibrio anguillarum* 90-11-287 (Skov *et al.*, 1995) was frequently used as test organism in agar based assays to evaluate production and activity of antagonistic compounds by *Pseudoalteromonas* strains. The ability of 51 strains to consistently inhibit *V. anguillarum* in a live cell assay (Vynne *et al.*, 2011) suggests that these antagonistic *Pseudoalteromonas* strains might present interesting opportunities for research and industrial applications within aquaculture probiotics. In spite of the pathogenic traits of some Pseudoalteromonads, they are not considered a problem within aquaculture where e.g. *Vibrio* species will typically pose a greater problem (Toranzo *et al.*, 2005). This opens the possibility for evaluating *Pseudoalteromonas* as fish probiotics on a case-by-case basis. Ideally non-pathogenic strains would be pursued. However, pathogenic traits may be specific towards certain species of fish or mollusks which may allow for selective use of for instance an algal pathogen in turbot aquaculture.

### **3.4 PATHOGENICITY OF PSEUDOALTEROMONADS**

Whether discussing biotechnological applications or the ecology of *Pseudoalteromonas* strains, it is prudent to consider their potential for pathogenic interactions. The majority of *Pseudoalteromonas* strains are unlikely to be human pathogens as they do not grow well at 37°C, and there are no literature reports of infectious disease in humans caused by *Pseudoalteromonas* bacteria. While not strictly a result of pathogenicity, the poisoning that can occur when eating

certain fish of the order Tetradontiformes is attributed to an accumulation of diverse marine bacteria that produce the neurotoxin tetrodotoxin (Chau *et al.*, 2011;Noguchi *et al.*, 2006). Among these, *Pseudoalteromonas tetraodonis* was isolated from the skin slime of a pufferfish and produces tetrodotoxin (Simidu *et al.*, 1990), hence it is likely involved in human poisoning following consumption of pufferfish. *Pseudoalteromonas* species also colonize and grow on ice-stored fish (Broekaert *et al.*, 2011) but are unlikely to be a cause of infectious disease since they do not grow at human body temperature (37C). Going beyond the human perspective and into the natural marine environment of *Pseudoalteromonas* bacteria, a number of *Pseudoalteromonas* species are associated with infectious disease in fish, molluscs or seaweed and alga. As the name suggests, *P. piscicida* is a fish pathogen. Rapid killing was observed in challenge tests involving live bacterial culture (Bein, 1954). No attempts were made to identify the cause of killing, but no fish death was observed when using a different unidentified marine bacterium in the same experimental setup. A different strain of *P. piscicida* infects damselfish eggs causing significantly higher mortality than untreated controls or common fish pathogen *Vibrio anguillarum* (Nelson & Ghiorse, 1999). *P. piscicida* is known to produce several bioactive substances and one might speculate that the cytotoxic compound norharman could be involved in pathogenicity (Zheng *et al.*, 2006). *Pseudoalteromonas* sp. LT-13 may be an opportunistic pathogen, taking advantage of the weakening that occurs upon infection in scallop larvae (Sandaa *et al.*, 2008). An earlier study suggested *Pseudoalteromonas* sp. LT-13 acted as a true pathogen (Torkildsen *et al.*, 2005), however this may have been due to a general weakening of the scallop larvae due to the high bacterial load encountered in the experimental setup. A follow-up study using lower bacterial concentrations indicated that LT-13 is not a primary pathogen, but may be involved in infections as an opportunistic secondary pathogen (Sandaa *et al.*, 2008). *Pseudoalteromonas* species have been isolated in numerical abundance from diseased pacific oysters (Garnier *et al.*, 2007), but no direct evidence of infection was obtained through re-inoculation of a healthy population with a pure bacterial culture. *P. elyakovii* was isolated from diseased spots on *Laminaria japonica* (Sawabe *et al.*, 2000) and was suggested to cause the disease.

The limited number of literature reports on pathogenic *Pseudoalteromonas* species suggests that these bacteria do not represent a danger to human health, and only rarely are involved in eukaryote disease.

### 3.5 BIOACTIVE COMPOUNDS PRODUCED BY *PSEUDOALTEROMONAS*

The wide occurrence of *Pseudoalteromonas* shows that members of this genus are successful in colonizing and persisting in a broad range of habitats. Bioactive substances such as enzymes, antimicrobials and antifouling cues are likely part of the reason for this success, enabling the producer strains access to nutrients or advantages through inhibition of competing microbes and modulation of the behavior of macroscopic animals. The bioactive molecules can be roughly divided into two groups: Small-molecule (molecular weight of less than ca. 3 kDa) non-proteinaceous compounds that are products of secondary metabolism and proteinaceous compounds including both large enzyme complexes and small peptides. The chemical diversity of the compounds is staggering when considering compounds with antibacterial or cytotoxic action, making *Pseudoalteromonas* strains interesting targets for discovery efforts focused on bioactive compounds with both pharmaceutical and industrial applications. Here, an overview of the known bioactive compounds produced by *Pseudoalteromonas* strains is presented (table 4, see page 36). Enzyme activity and modulation of eukaryote behavior are covered in separate paragraphs. *P. luteoviolacea* will have a dedicated bioactivity paragraph, as this species has been a focal point of this Ph.D. work.

#### 3.5.1 NON-LUTEOVIOLACEA *PSEUDOALTEROMONAS*

Antibacterial activity is observed in multiple species of *Pseudoalteromonas*. The inhibitory spectrum varies however, as some species inhibit Gram-negative or Gram-positive bacteria exclusively while others have very broad or very narrow ranges of inhibition. An example of this is *P. phenolica* O-BC30 which produces novel structurally related anti-MRSA compounds. The compound MC21-A was bacteriocidal against MRSA *Staphylococci* and exhibited broad-spectrum antibacterial activity (Isnansetyo & Kamei, 2003) while MC21-B inhibited Gram-positive strains but not Gram-negative bacteria or fungi (Isnansetyo & Kamei, 2009). Similarly *Pseudoalteromonas* sp. F-420 produces the antibiotic korormicin, which is active against Gram-negative marine bacteria (Yoshikawa *et al.*, 1997). The compound targets a unique NADH:quinone reductase (Yoshikawa *et al.*, 1999) found in many marine bacteria which enables the bacteria to maintain a sodium-motive force under the conditions present in the marine environment (Unemoto & Hayashi, 1993). This

makes korormicin a highly specialized substance which targets marine bacteria, and more knowledge of its in situ role in ecology would be of great interest. Another interesting case are the structurally distinct class of small molecule antibiotic compounds called thiomarinols, produced by *Pseudoalteromonas* sp. SANK 73390 (Shiozawa *et al.*, 1993; Shiozawa *et al.*, 1995; Shiozawa *et al.*, 1997). Thiomarinols are active against both Gram-negative and Gram-positive bacteria, and are synthesized from the products of two biosynthetic pathways located on a plasmid (Fukuda *et al.*, 2011). Synthetic analogues have been engineered by mutagenesis of the producer bacterium (Murphy *et al.*, 2011). *Pseudoalteromonas* sp. CMMED 290 produces pentabromopseudilin (PBP), bromophene and two novel anti MRSA compounds structurally related to PBP and bromophene respectively (Fehér *et al.*, 2010). This is a good example of how biosynthetic intermediates may be isolated as interesting bioactive lead compounds based on bioassays which likely do not reflect the ecological role of the active compound. Prodigiosin and related compounds feature prominently in several bacteria, notably *Serratia marcescens*. Among the *Pseudoalteromonads*, *P. denitrificans* (Kawauchi *et al.*, 1997; Kim *et al.*, 1999) and *P. rubra* (Fehér *et al.*, 2008; Gerber, 1971; Vynne *et al.*, 2011) produce the red pigments prodigiosin and cycloprodigiosin HCl (figure 14).



**Figure 14:** Fractionated ethyl acetate extracts of *P. rubra* strains (Maria Månsson)

This class of compounds has been investigated for several pharmacological effects as reviewed by (Bennett & Bentley, 2000), for example cycloprodigiosin has potential applications as an antimalarial (Kawauchi *et al.*, 1997) and immunosuppressive agent (Kim *et al.*, 1999). Recent research has shown that prodigiosin is a potential insecticide with activity against mosquito larvae

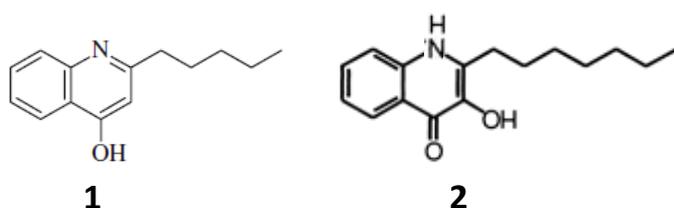
(Patil *et al.*, 2011), while others have provided evidence that prodigiosin functions as a UV-protective pigment which may be the actual reason for its wide-spread occurrence in marine bacteria (Boric *et al.*, 2011).

Some *Pseudoalteromonas* species produce antifungal compounds which are speculated to have a role in surface colonization. *P. issachenkonii* produces the antifungal compound isatin (Kalinovskaya *et al.*, 2004), which was suggested to confer a competitive advantage in colonizing algal thalli which is the natural niche of the producer species. Isatin-producing *Alteromonas* bacteria have also been isolated from shrimp embryos and it was shown that the *Alteromonas* bacteria protected the embryos from infection by a pathogenic fungus (Gil-Turnes *et al.*, 1989). *P. tunicata* produces an antifungal tambjamine compound structurally related to prodigiosin (Burke *et al.*, 2007). This is likely the compound that was responsible for the antifungal activity observed in co-culture biofilms of *P. tunicata* and a marine fungus, where *P. tunicata* was able to exclude the fungus from the biofilm (Franks *et al.*, 2006).

*P. maricaloris* KMM 636 produces the chromopeptides bromoalterochromide A and A' which are cytotoxic towards sea urchin eggs (Speitling *et al.*, 2007). We identified bromoalterochromide A in 12 strains of *P. flavipulchra* which are closely related to *P. maricaloris* and *P. piscicida*, and in one *P. phenolica* strain and one *P. rubra* strain. From these strains we also identified what is likely two novel bromoalterochromides, that were also produced in 2 strains of *P. aurantia* / *P. citrea* (Vynne *et al.*, 2011). Synthesis of bromoalterochromides is shared among several *Pseudoalteromonas* species, and the cytotoxic effect of bromoalterochromides A and A' provides evidence for the involvement of bromoalterochromides in bacterial-eukaryote interactions. Unfortunately, bromoalterochromides degrade rapidly upon exposure to UV light which makes them hard to purify and detect.

The antibacterial compound 2-*n*-pentyl-4-quinolinol was identified from a *Pseudoalteromonas* strain related to *P. citrea* and *P. aurantia* (Long *et al.*, 2003), and from *P. piscicida* A1-J11 (del Castillo *et al.*, 2008). During my Ph.D. work, the compound was identified in 5 of 5 strains related to *P. citrea* and *P. aurantia*, and in 1 of 13 strains related to *P. flavipulchra* and *P. piscicida* (Vynne *et al.*, 2011). An additional 3 quinolinone compounds were also identified in some of these strains, with no apparent species-specific pattern. 2-*n*-pentyl-4-quinolinol strongly inhibited *Vibrio harveyi*

and *Vibrio fischeri* but not other *Vibrio* sp. or *Pseudomonas* sp. (Long *et al.*, 2003). Interestingly, it was previously described as a primary antibiotic in a marine *Pseudomonad* (Wratten *et al.*, 1977) and as seen in figure 15 resembles the structure of the quinolone molecules utilized for signalling by *Pseudomonas aeruginosa* (Pesci *et al.*, 1999) which opens for discussion of the role of small-molecule antibiotics in the environment.



**Figure 15:** Chemical structure of 2-*n*-pentyl-4-quinolinol **1** and the *Pseudomonas* quinolone signal 2-heptyl-3-hydroxy-4-quinolone **2**.

Recent publications have raised questions regarding the role of antimicrobial compounds *in situ*. Many antibiotics have profound influence on gene expression levels in microorganisms exposed to sub-lethal concentrations of the antibiotics (Davies *et al.*, 2006), for instance subinhibitory concentrations of  $\beta$ -lactam or aminoglycoside antibiotics induce biofilm formation in *Pseudomonas aeruginosa* (Bagge *et al.*, 2004; Hoffman *et al.*, 2005). Biofilm formation is known to increase bacterial resistance towards antibiotics (Mah & O'Toole, 2001), and thus may be considered a defense mechanism. Indeed, laboratory studies indicate that antibiotic production may be of particular benefit in a structured environment (Chao & Levin, 1981) which may also be applicable for marine surface colonizers. Little is known of the effect of an antibiotic on the producer organism itself and the microbial community as a whole in a marine natural environment, and exploration of the *in situ* activities of naturally occurring antibiotics has the potential to shed a new light on marine microbial ecology.

**Table 4:** Bioactive compounds produced by *Pseudoalteromonas* species. Modified from Bowman (2007)

Species	Source	Pigment	Bioactive compounds	Bioactivity
<i>P. aliena</i>	Seawater	+ (melanin)	Unknown compounds	Anti-tumorogenic
<i>P. agarivorans</i>	Seawater, ascidians	-	Unknown	Degrades algal polysaccharides
<i>P. antarctica</i>	Seawater, sea-ice, muddy soils	-	-	Cold-active enzymes
<i>P. arctica</i>	Seawater	-	-	-
<i>P. atlantica</i>	Seawater, marine alga	-	-	Degrades algal polysaccharides
<i>P. aurantia</i>	Seawater, alga	Yellow	2-pentyl-4-quinolinol Macromolecule Unknown	Antibacterial Antibacterial Antidiatom
<i>P. byunsanensis</i>	Tidal flat sediment	-	-	-
<i>P. carrageenovora</i>	Seawater, marine alga	-	-	Degrades algal polysaccharides
<i>P. citrea</i>	Seawater, mussels, ascidians, sponges	Yellow, melanin	2-pentyl-4-quinolinol Unknown	Antibacterial Antidiatom
<i>P. denitrificans</i>	Seawater	Red	Cycloprodigiosin HCl  Unknown substance	Antimalarial, immunosuppressant, anti-tumorogenic Induces settlement of sea urchin <i>Heliocidaris erythrogramma</i>
<i>P. distincta</i>	Sponge	(melanin)	-	-
<i>P. donghaensis</i>	Seawater	-	-	-
<i>P. elyakovii</i>	Mussels, alga	-	-	-
<i>P. espejana</i>	Seawater	-	-	-
<i>P. flavipulchra</i>	Seawater, copepod	Orange/yellow	2-pentyl-4-quinolinol	Antibacterial
<i>P. haloplanktis</i>	Seawater, scallop	-	Isovaleric acid Unknown	Antibacterial Antidiatom Cold-active enzymes
<i>P. issachenkonii</i>	Alga	-	Isatin	Antifungal, hemolytic
<i>P. lipolytica</i>	Estuarine water	-	-	-
<i>P. luteoviolacea</i>	Seaweed, algae, rocks, seawater	Purple	Violacein  Pentabromopseudilin  Indolmycin L-amino acid oxidase 2,4-Dibromo-6-chlorophenol	Antibacterial, cytotoxic Antibacterial, inhibits myosin motor activity Antibacterial Antibacterial Antibacterial, antifungal
<i>P. maricaloris</i>	Sponges	Yellow	Bromoalterochromides A and B	Antibacterial, cytotoxic towards sea urchins
<i>P. marina</i>	Tidal flat sediment	-	-	-

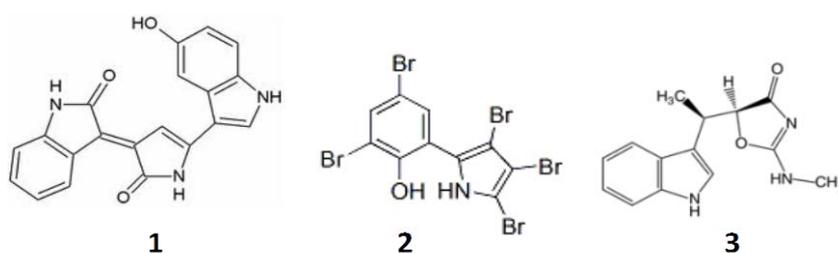
<i>P. mariniglutinosa</i>	Diatoms	-	-	-
<i>P. nigrifaciens</i>	Seawater, raw fish, salted foods, mussels	(melanin)	-	-
<i>P. paragorgicola</i>	Sponge	-	-	-
<i>P. peptidolytica</i>	Seawater	Yellow	Unknown compounds	Antimicrobial, hemolytic
<i>P. phenolica</i>	Seawater	Brown	2,2',3-tribromobiphenyl-4-4'-dicarboxylic acid 3,3',5,5'-tetrabromo-2,2'-biphenyldiol	Antibacterial against Gram-positive bacteria Antibacterial
<i>P. piscicida</i>	Estuarine water, fish samples, copepods	Yellow	Protein 2- <i>n</i> -pentyl-4-quinolinol Unknown	Antibacterial Antibacterial Antidiatom
<i>P. prydzensis</i>	Seawater	-	-	-
<i>P. rubra</i>	Seawater	Red	Prodigiosin, cycloprodigiosin, 2-( <i>p</i> -hydroxybenzyl)prodigiosin Rubrenoic acids Unknown	Antimalarial, immunosuppressant, insecticide, UV-protection Bronchodilatory Antidiatom
<i>P. ruthenica</i>	Shellfish, seawater	Pale orange	Unknown compounds	Antimicrobial
<i>P. spongiae</i>	Sponge	Pale orange	Unknown compounds	Strong induction of <i>Hydroides elegans</i> settlement
<i>P. tetraodonis</i>	Puffer fish	-	Tetrodotoxin	Neurotoxic
<i>P. translucida</i>	Seawater	-	-	-
<i>P. tunicata</i>	Tunicate	Dark green	Tambjamine Violacein AlpP L-amino acid oxidase Unknown Unknown protein	Kills <i>C. elegans</i> Antibacterial, cytotoxic Antibacterial, autoinhibitory Antidiatom Rapidly kills <i>C. elegans</i>
<i>P. ulvae</i>	Marine alga	Purple	Unknown Unknown	Inhibits settlement of invertebrate larvae and algal spores Antidiatom
<i>P. undina</i>	Seawater, fish	-	Unknown	Antidiatom, hemolytic
<b><i>Pseudoalteromonas</i> sp. :</b>				
SANK 73390			Thiomarinols A, B, C, D, E, F, G	Antibacterial
F-420	Alga	White / yellow	Korormicin	Antibacterial against Gram-negative bacteria
SWAT5	Marine particle	Unknown	2- <i>n</i> -pentyl-4-quinolinol	Antibacterial
S2V2	Seawater	White / yellow	Non-proteinaceous	Antibacterial

Unknown (a)	Seaweed	Unknown	Halophenol	Antibacterial, antifungal
CMMED 290	Nudibranch	Unknown	2,3,5,7-tetrabromobenzofuro- [3,2- <i>b</i> ]pyrrole	Anti MRSA
			4,4',6-tribromo-2,2'-biphenol	Anti MRSA
			Pentabromopseudilin	Antibacterial, inhibits myosin motor activity
			Bromophene	Antibacterial
J010	Corraline algae	Unknown	Tetrabromopyrrole	Induces settlement and metamorphosis of coral larvae

(a) Jiang *et al.* 2006

### 3.5.2 PSEUDOALTEROMONAS LUTEOVIOLACEA

One of the first antibacterial compounds that were described from marine bacteria was pentabromopseudilin (PBP), identified as a novel compound produced by a bacterium later assigned to the *Pseudoalteromonas* (Burkholder *et al.*, 1966; Fenical, 1993). This compound was identified in a subset of *P. luteoviolacea* strains in this Ph.D. work (figure 16), and although it was claimed to also be produced by a *Chromobacterium* sp. (Andersen *et al.*, 1974) the bacterial strain in question shows biochemical characteristics resembling *P. luteoviolacea* over *Chromobacterium*, in particular a negative catalase reaction, survival for 3-4 days in plate cultures and optimum growth around room temperature (Gauthier, 1982; Gauthier & Flatau, 1976).



**Figure 16:** Small-molecule bioactive compounds produced by *P. luteoviolacea*. Violacein **1**, pentabromopseudilin **2** and indolmycin **3**.

PBP shows excellent *in vitro* activity against Gram-positive bacteria but is not active against Gram-negative bacteria or *Candida albicans* (Burkholder *et al.*, 1966). PBP is a potent inhibitor of myosin motor activity with clinical potential in treatment of malaria, cancer and heart disease (Fedorov *et*

*al.*, 2009). In addition to PBP, *P. luteoviolacea* produces the purple pigment violacein. Violacein was reported to have antibacterial activity (Lichstein & Vandesand, 1945), though this was not observed in this Ph.D. work in tests against *P. luteoviolacea* strains, *Vibrio anguillarum* and *Staphylococcus aureus*. Violacein has potent cytotoxic activity (da Silva Melo *et al.*, 2000), and is likely used as a chemical defense molecule by the producing organisms (Matz *et al.*, 2004; Matz *et al.*, 2008). Violacein production is also described in *P. tunicata* (Matz *et al.*, 2008), *P. denitrificans* (Yada *et al.*, 2008) and in several other genera (Agematu *et al.*, 2011; Becker *et al.*, 2009; Hakvåg *et al.*, 2009; Lichstein & Vandesand, 1945; Wang *et al.*, 2009). Phylogenetic analysis of amino acid sequences of biosynthetic proteins involved in violacein synthesis indicate that the violacein biosynthesis in Pseudoalteromonads diverged at an early point (Hakvåg *et al.*, 2009). This may be the result of a genetic transfer event involving ancestral bacteria which since differentiated into *Pseudoalteromonas* species and violacein producing  $\beta$ -Proteobacteria, respectively, or it could indicate that violacein biosynthesis is an ancient pathway which originated in a common ancestor.

A third small-molecule antibiotic substance produced by *P. luteoviolacea* is indolmycin (Månsson *et al.*, 2010; Vynne *et al.*, 2011), as described in this Ph.D. work. Hitherto known only from *Streptomyces* species (Kanamaru *et al.*, 2001; von Wittenau & Els, 1961) indolmycin is a potent antibacterial agent with selective antibacterial activity against both Gram-negative and Gram-positive bacteria and particularly effective against *S. aureus* with a reported minimum inhibitory concentration of 0.25  $\mu\text{g/ml}$  (Hurdle *et al.*, 2004) and *Helicobacter pylori* (Kanamaru *et al.*, 2001). The tryptophan-derived indolmycin inhibits the bacterial tryptophanyl-tRNA synthetase (Werner *et al.*, 1976) by competitive binding, and mutations leading to changes of specific amino acids in the tryptophanyl-tRNA synthetase or changes in the promotor sequence of the tryptophanyl-tRNA synthetase gene leads to high levels of indolmycin resistance in *Streptomyces coelicolor* and *E. coli* (Kitabatake *et al.*, 2002; Vecchione & Sello, 2009). A *P. luteoviolacea* with no strain name given was reported to produce the antibacterial and antifungal compound 2,4-Dibromo-6-chlorophenol (Jiang *et al.*, 2000).

In the course of this Ph.D. study a link between phylogeny based on housekeeping genes and production of the bioactive compounds indolmycin and PBP was discovered (Vynne *et al.*, 2012b).

Indolmycin or PBP were produced by distinct phylogenetic groups, providing evidence for the importance of these compounds in the producer organisms as the biosynthetic pathways are maintained over evolutionary time.

In addition to the small-molecule compounds mentioned above, *P. luteoviolacea* is capable of synthesizing a macromolecular antibacterial substance. An L-amino acid oxidase (LAO) was characterized from two *P. luteoviolacea* strains by (Gomez *et al.*, 2008) and is likely synonymous with the macromolecule previously described in the type strain (Gauthier, 1976). Other LAOs are known from marine bacteria (Lucas-Elio *et al.*, 2005) and includes the AlpP autolysis protein produced by *P. tunicata* (Mai-Prochnow *et al.*, 2008) and an anti-MRSA agent produced by *P. flavipulchra* (Chen *et al.*, 2010). It is tempting to speculate that autolysis in *P. luteoviolacea* is due to LAO activity (Gauthier & Flatau, 1976), as suggested for *P. tunicata* (Mai-Prochnow *et al.*, 2004). The physiological role of LAOs in marine bacteria is unknown, though the antimicrobial effect of LAOs is due to the release of hydrogen peroxide when the LAO substrate is oxidized (Gomez *et al.*, 2008; Mai-Prochnow *et al.*, 2008). In addition to possible *in situ* antimicrobial effects they may increase available nitrogen by oxidizing amino acids in the local milieu as suggested for fungal LAOs (Davis *et al.*, 2005).

### **3.6 ENZYMATIC ACTIVITIES OF PSEUDOALTEROMONADS**

Microbial life is dependent on enzyme activities. Extracellular enzymes play crucial roles in providing accessible nutrient sources and are often highly specialized for the niches of the producer organism. This ecology-driven specialization has gone on for the entirety of microbial evolution, and thus offers a wealth of highly specialized enzymes which may have uses within industries as diverse as dairy production and biofuels. The ubiquitous nature of the Pseudoalteromonads is an indication that they thrive in a wide range of environments, and arctic strains in particular have been investigated for enzymatic activities in the expectation that novel enzymes active at low temperature would be discovered. An overview of some of the currently described enzymatic activities is presented in table 5.

Many of the characterized enzymes from *Pseudoalteromonas* strains are from psychrophilic strains and exhibit cold-adapted properties. This opens for different applications such as treatment of dairy products to remove lactose at low temperature (Hoyoux *et al.*, 2001), however the cold

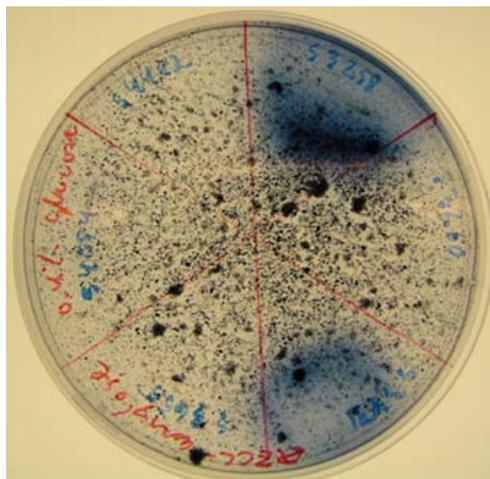
adapted enzymes are often also less stable and will denature or undergo conformational changes at a lower temperature than mesophilic enzymes (Georlette *et al.*, 2004).

**Table 5:** Enzyme activity from *Pseudoalteromonas* strains

Strain	Product	Reference
<i>P. haloplanktis</i>	Cold-adapted $\beta$ -galactosidase	Hoyoux <i>et al.</i> 2001
<i>Pseudoalteromonas</i> sp.	Cold-adapted $\beta$ -galactosidase	Fernandes <i>et al.</i> 2002
<i>P. haloplanktis</i> TAC125	Lipase PhTAC125 Lip1	de Pascale <i>et al.</i> 2008
<i>Pseudoalteromonas</i> sp. DY3	Cold-active cellulase	Zeng <i>et al.</i> 2006
<i>Pseudoalteromonas</i> sp. NJ276	Cold-active protease	Wang <i>et al.</i> 2008
<i>Pseudoalteromonas</i> sp. SM9913	Cold-adapted subtilase	Yan <i>et al.</i> 2009
<i>P. haloplanktis</i> TAB23	Cold-adapted $\alpha$ -amylase	Tutino <i>et al.</i> 2002
<i>P. haloplanktis</i> ANT/505	Pectate lyase	Van Truong <i>et al.</i> 2001
<i>P. haloplanktis</i>	Xylanase	Van Petegem <i>et al.</i> 2002
<i>Pseudoalteromonas</i> sp. MB-1	Endoglucanase	You <i>et al.</i> 2005
<i>Pseudoalteromonas</i> sp. 005NJ	$\alpha$ -l-rhamnosidase	Gaston Orrillo <i>et al.</i> 2007
<i>Pseudoalteromonas arctica</i>	Cold-adapted esterase	Al Khudary <i>et al.</i> 2010
<i>P. haloplanktis</i>	Cold-adapted cellulase	Violot <i>et al.</i> 2005
<i>P. haloplanktis</i>	DNA ligase	Georlette <i>et al.</i> 2000
<i>P. carrageenovora</i>	Carrageenase	Michel <i>et al.</i> 1999
<i>Pseudoalteromonas</i> sp. S9	Chitinase	Techkarnjanaruk <i>et al.</i> 1997
<i>P. arctica</i> GS230	Cold-adapted $\alpha$ -amylase	Lu <i>et al.</i> 2010
<i>P. antarctica</i> N-1	Agarase	Vera <i>et al.</i> 1998
<i>Pseudoalteromonas</i> sp. CKT1	Agarase	Xavier Chiura <i>et al.</i> 2000

Representative strains from the collection used in the present work were screened for production of industrially relevant enzymes in an agar based assay (figure 17 below) containing azurine cross-linked polysaccharides (Megazyme, Ireland). Six of 12 strains tested amylase positive and 5 of 12 tested curdlanase positive, with four strains testing positive in both assays (Vynne, unpublished).

**Figure 17:** Amylase assay on *Pseudoalteromonas* strains. The AZCL-substrate is seen as blue particles in the agar and dark blue halos indicate enzyme activity.



The positive strains were both pigmented and non-pigmented. A reason for the low number of observed enzymatic activities within the *Pseudoalteromonas* strains from this Ph.D. study could be that pigmented strains were overrepresented in the selection for screening, since the pigmented bacteria were the primary research interest due to their antagonist activities while the majority of the producer organisms listed in table 5 are of the non-pigmented clade. Also, since the screening took place at 25°C, some strains may have cold-adapted enzyme activities which were not detected due to the incubation temperature.

### **3.7 ANTI-FOULING POTENTIAL OF *PSEUDOALTEROMONAS* SPECIES**

While some *Pseudoalteromonas* promote settlement of eukaryotes as previously described (Huggett *et al.*, 2006), many species have been investigated for their anti-fouling and anti-biofilm activities (Bernbom *et al.*, 2011; Boyd *et al.*, 1999; Dobretsov & Qian, 2002; Egan *et al.*, 2001a; Holmström *et al.*, 2002). Fouling is a build-up of biological material on a surface, which in the marine environment develops rapidly unless preventive measures are taken. Briefly, submerging a clean surface into the marine environment will cover the surface in a thin film of organic substances within minutes. Bacteria and diatoms will follow within hours, forming the basis for increased colonization by larvae of macrofouling organisms (Abarzua & Jakubowski, 1995; Colwell, 1983; Wahl, 1989). Fouling is a problem in the marine industries, particularly within shipping as a dense layer of fouling rapidly develops on a submerged hull reducing speed and fuel efficiency unless preventive measures are taken (Champ, 2000).

*Pseudoalteromonas tunicata* D2 produces anti-fouling compounds (Holmström *et al.*, 1998) shown to correlate with pigment production (Egan *et al.*, 2002). Other *Pseudoalteromonas* strains inhibit larvae and diatom settlement in direct cell interaction assays (Lee & Qian, 2003) and in tests of aqueous bacterial cell extracts (Dobretsov & Qian, 2002). Some antifouling effects may be due to bacterial antagonism towards other microorganisms which promote larval settlement, but there is no consistent link between antifouling and antibacterial compounds (Bernbom *et al.*, 2011).

### 3.8 CONCLUSION FROM CHAPTER 3

The genus *Pseudoalteromonas* has a complex and intricate taxonomy and underlying phylogeny. Care should be taken if 16S rRNA gene sequences are used as the only means of species identification when a novel *Pseudoalteromonas* isolate is being described, as this Ph.D. thesis demonstrate that even at 99% 16S rRNA sequence identity, it remains possible to describe new species. Correct taxonomical assignments are important for scientific communication and understanding evolutionary dynamics with a bacterial genus. Many *Pseudoalteromonas* species produce bioactive compounds and the present study expanded on the knowledge of bioactive small-molecule compounds and their distribution within the *Pseudoalteromonas* species. The potent antibacterial compound indolmycin was discovered for the first time in marine bacteria, and several other known bioactives were identified. It was discovered that, within a single species, three antibiotics were produced by distinct phylogenetic groups. The selective maintenance of the biosynthetic pathways suggests the antibiotics play an important role for the producer organism, likely in community interactions and protection given the antagonist nature of the compounds. The role of antibiotics in the natural environment is increasingly up for debate, as sublethal concentrations of antibiotics may in fact have dramatic effects on bacterial populations and hence what we perceive as antibiotics may in some cases serve a different purpose under natural conditions. As novel *Pseudoalteromonas* species and strains with interesting bioactivities are continuously described, it stands to reason that there is a rich untapped potential for biodiscovery of novel bioactive substances within the Pseudoalteromonads.

## 4.0 QUORUM SENSING IN REGULATION OF BACTERIAL PRODUCTION OF ANTIMICROBIAL COMPOUNDS

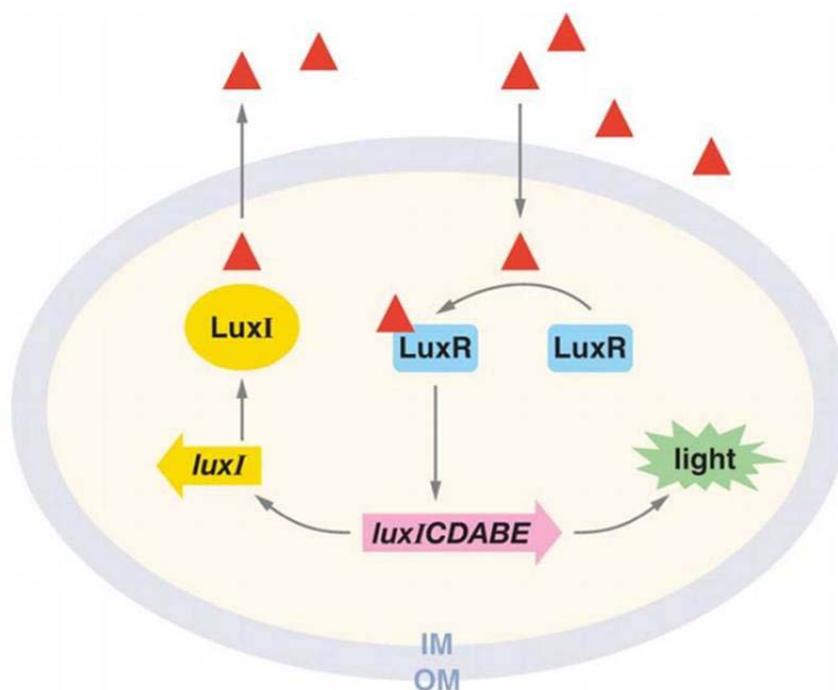
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Traditionally, bacteria were thought of as individual cells with no means to actively coordinate a change in the population of gene regulation and phenotype. As our understanding of bacterial behavior in both pure cultures and mixed communities has increased, it has become clear that intra- and inter-species cell-cell communication play a key role in coordinating gene expression and subsequently phenotype in both pure cultures and mixed communities. There are many types of bacterial communication (Blango & Mulvey, 2009; Dubey & Ben-Yehuda, 2011), however, one of the currently most studied is often referred to as quorum sensing (QS). The name quorum sensing reflects its reliance on cell numbers, and it is now known to regulate advanced phenotypes such as bioluminescence (Nealson & Hastings, 1979), virulence (Latifi *et al.*, 1995), motility (Huber *et al.*, 2001; Kohler *et al.*, 2000), biofilm formation (Labbate *et al.*, 2004) and antibiotic production (Berger *et al.*, 2011; Duerkop *et al.*, 2009; Hothersall *et al.*, 2011). QS is mediated by small-molecule chemical compounds called auto-inducers, which diffuse freely into the extracellular environment and act as signal carriers. As part of the work with antibiotic producing *Pseudoalteromonas*, we discovered compounds that possibly could be autoinducers and hypothesized that these were involved in regulation/production of a particular antibiotic. This chapter outlines the general principles of QS and focuses on examples in which bacterial antibiotic production is QS regulated.

### 4.1 THE *VIBRIO FISCHERI* LUXI/LUXR QUORUM SENSING MODEL

The process of QS has been described in many bacterial species and regulates diverse physiological and chemical processes (see e.g. (Dobretsov *et al.*, 2009; Van Houdt *et al.*, 2007). Quorum sensing was first described in the marine bacteria *Vibrio fischeri* where it regulates bioluminescence (Nealson & Hastings, 1979). This cell density dependent QS system is studied in depth and forms the canonic Lux-based QS system (figure 18). Similar LuxIR-like QS systems have been widely described in Gram-negative bacteria (Whitehead *et al.*, 2001) and are characterized by containing homologues to the LuxI and LuxR proteins. Four main components are involved in LuxIR QS: the

LuxI AHL synthetase, the acylated homoserine-lactone (AHL) signaling molecules, the LuxR transcriptional regulator and the genes encoding the QS regulated phenotype (Miller & Bassler, 2001). The LuxI protein is responsible for synthesis of the AHL molecules and even at low cell densities individual cells produce a small amount of the cognate AHL (Engebrecht & Silverman, 1984). As cell density increases, the local concentration of AHL rises and eventually reach a threshold concentration which activates the LuxIR regulatory system. The system is activated by LuxR proteins binding the cognate AHL, which induces a conformational change that allows the DNA binding domain of the LuxR protein to recognize and bind to a specific DNA sequence called a *lux*-box (Choi & Greenberg, 1992; Welch *et al.*, 2000; Zhu & Winans, 2001). Upon LuxR binding, transcription of the genes downstream of the *lux*-box is activated. The *luxI* gene may also be situated downstream of the LuxR binding site, which upon LuxR binding leads to an increased production of LuxI and subsequently increased AHL concentration creating a positive feedback loop. Several variations on this central concept exist, for instance LuxR can focus as a repressor protein (Fuqua *et al.*, 1996).

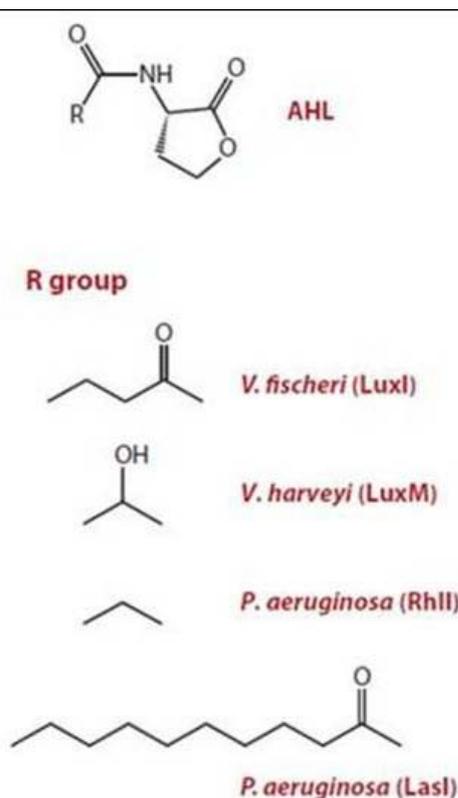


**Figure 18:** The canonical LuxI/LuxR QS system in *V. fischeri*. IM, inner membrane; OM, outer membrane. Adapted from Waters & Bassler (2005).

#### 4.1.1 ACYLATED HOMOSERINE-LACTONES: STRUCTURE AND ANALYSIS

The AHL molecules are the active signal carrying molecules in LuxIR quorum sensing. Most diffuse freely in and out of the individual bacterial cells, enabling the AHL-producing bacteria – and possible “eavesdroppers” – to probe the concentration of signal molecules and monitor the microbial environment to respond in a coordinated, social way. Figure 19 shows the typical AHL compositions consisting of a homoserine lactone ring with a C<sub>4</sub> – C<sub>18</sub> N-acyl side chain (Fuqua *et al.*, 2001).

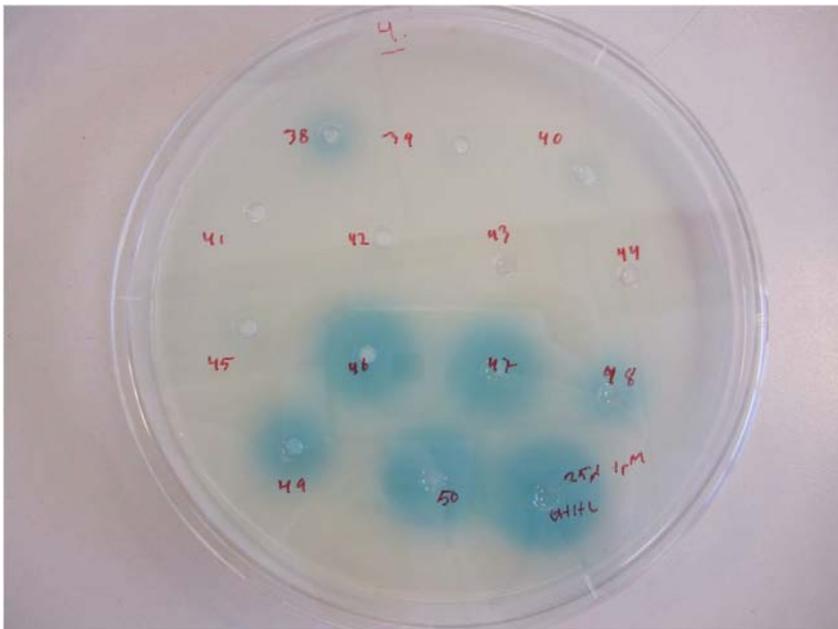
**Figure 19:** Acylated homoserine lactones produced by different bacterial species and recognized by corresponding LuxR homologues. The R side chain may also contain a number of double bonds. Adapted from Ng & Bassler (2009).



The lactone ring will open at alkaline pH, eliminating the signaling properties of the AHL. Susceptibility to this lactonolysis decrease as the N-acyl chain increases in length (Yates *et al.*, 2002). AHLs commonly have 3-oxo or 3-hydroxy substitutions, which influences signal specificity, and may have a number of unsaturated double bonds in the side chain. The biosynthesis of AHLs rely on cellular pools of acylated acyl carrier protein and S-adenosyl-L-methionine which are the substrates used by the LuxI AHL synthetase (Parsek *et al.*, 1999; Watson *et al.*, 2002). A very

interesting recent study identified a new class of homoserine lactone QS signal molecules which are based on *p*-coumaric acid rather than cytoplasmic fatty acids and function in a LuxIR analogue QS system (Schaefer *et al.*, 2008).

AHLs are commonly identified via bioassays that rely on well characterized QS systems that detect AHL signals with varying sensitivity and specificity (Cha *et al.*, 1998; McClean *et al.*, 1997; Ravn *et al.*, 2001; Winson *et al.*, 1998). Often, these systems are engineered to function as biosensors, e.g. through a *lacZ* or luminescence reporter fusion (figure 20). Since the assays are based on naturally occurring pathways, a given biosensor will in most cases produce a stronger response when its cognate AHL is added, and the range of AHLs recognized varies among biosensors (Fekete *et al.*, 2007). The biosensors are also used in conjunction with methods from analytical chemistry in order to purify and identify the AHL compounds following bioassay guided fractionation strategies as described in chapter 1 but using AHL biosensors as indicator strains (Fekete *et al.*, 2007; Wang *et al.*, 2011).

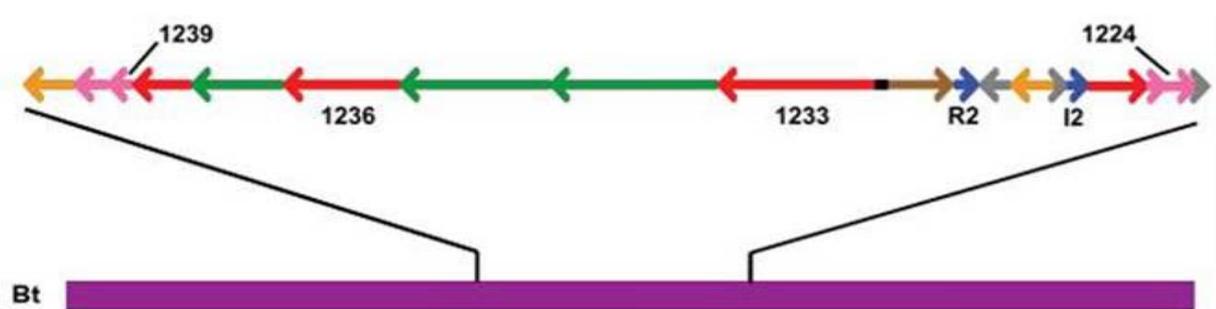


**Figure 20:** Bioassay for detection of AHLs. The monitor strain is *Agrobacterium tumefaciens* pZLR4. pZLR4 contains the Lux homologue Tra-system with a *lacZ* fusion. The blue zones indicate that the Tra-system was activated and *lacZ* transcribed, leading to production of a blue pigment due to specific cleavage of 5-bromo-4-chloro-indolyl- $\beta$ -D-galactopyranoside (Cha *et al.*, 1998). The samples tested were C-18 fractions of an ethyl acetate extract of *P. luteoviolacea* S4054.

## 4.2 THE ROLE OF QUORUM SENSING IN REGULATING BACTERIAL PRODUCTION OF ANTIBIOTICS

One of the many phenotypes regulated by QS is, in some bacteria, the production of antibiotic compounds. The ability to regulate and coordinate production of an antibiotic compound is an obvious advantage to the producer organisms, e.g. in surface colonization where QS could restrict production of antibiotics to situations where a microbial community is established, cell density is high and action against competitors is needed.

The genus *Burkholderia* contains several species that utilize signaling via QS mechanisms. Among these, production of antibiotic compounds by *Burkholderia cepacia* and *Burkholderia thailandensis* is under QS regulation. The antibiotic bactobolin which is effective against both Gram-positive and Gram-negative bacteria was recently identified in *B. thailandensis* E264 (Seyedsayamdost *et al.*, 2010). A biosynthetic pathway was proposed (figure 21) (Seyedsayamdost *et al.*, 2010) and shown to be under LuxIR-homologue QS control with AHL production being essential for expression of antibacterial activity (Duerkop *et al.*, 2009). Bactobolin inhibited *Bacillus subtilis* which, like *B. thailandensis*, is a soil bacterium and the authors speculate that the QS regulated production may allow *B. thailandensis* to chemically defend itself during growth as microcolonies in the soil environment. Other LuxIR-homologue QS systems with high homology to QS systems in the closely related *Burkholderia mallei* and *Burkholderia pseudomallei* exist in *B. thailandensis* and are speculated to regulate virulence factors (Duerkop *et al.*, 2007; Duerkop *et al.*, 2008).



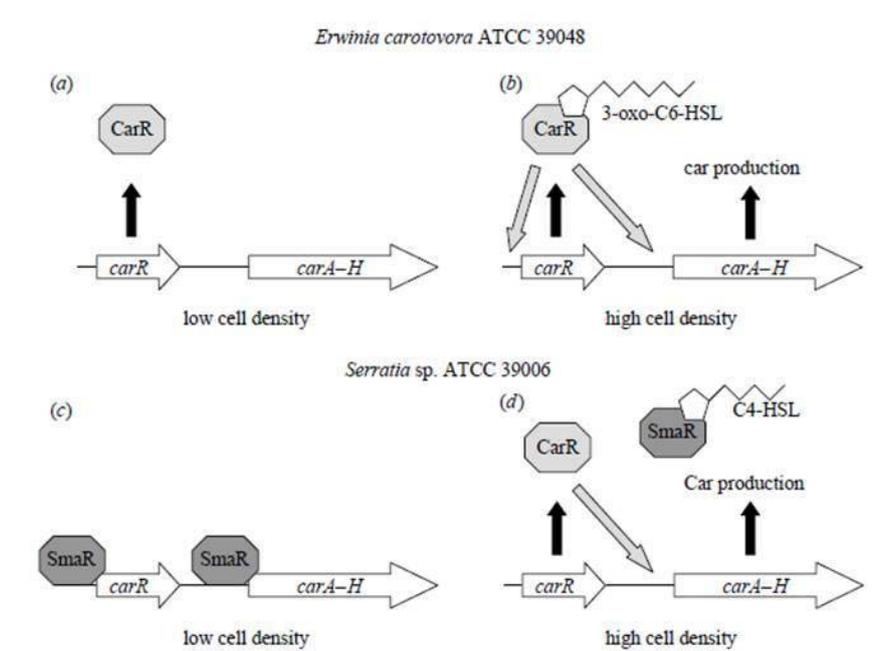
**Figure 21:** The QS regulated bactobolin pathway. I2 and R2 are LuxI and LuxR homologues, respectively. The red arrows are annotated as NRPS genes, green arrows indicate PKS genes, pink arrows are potential accessory antibiotic synthesis genes, orange arrows are putative transport genes, the brown arrow is a metallopeptidase, and gray indicates genes of unknown function. The numbers indicate genes targeted for molecular analyses. Modified from Duerkop *et al.* (2009)

The polyketide antibiotic mupirocin used in topical treatment of Staphylococci is produced by *Pseudomonas fluorescens* NCIMB 10586 and is under QS control (Thomas *et al.*, 2010). The genes *mupI* and *mupR* are regulatory genes with their products showing high amino acid homology to LuxI and LuxR, respectively (El-Sayed *et al.*, 2001). These genes are essential for biosynthesis of mupirocin and the MupI product was recently identified as 3-oxo-C10-HSL (Hothersall *et al.*, 2011). Interestingly, production of mupirocin was not increased by addition of exogenous 3-oxo-C10-HSL or *in trans* expression of *mupI*, suggesting that an additional control mechanism possibly related to the amidase homologue MupX influences production of mupirocin (Hothersall *et al.*, 2011). The involvement of the putative AHL-degrading enzyme MupX adds an extra level of complexity to this QS network and provides a glimpse of how tight regulatory control is employed by bacteria to ensure correctly timed production of, in this case, secondary metabolites with bioactivity.

The Gram-negative  $\gamma$ -Proteobacteria *Erwinia carotovora* and *Serratia* sp. ATTC 39006 produce the broad-spectrum  $\beta$ -lactam antibiotic carbapenem (Parker *et al.*, 1982). The production of carbapenem is under QS control in both bacteria (Bainton *et al.*, 1992; Thomson *et al.*, 2000) although interestingly the QS mechanisms are different with respect to the AHL binding molecules (figure 22 below). In *Erwinia*, the LuxR homologue CarR binds the AHL directly and subsequently activates transcription of the *car* biosynthetic pathway, in similar fashion as the canonical LuxIR system. In *Serratia* sp. ATTC 39006, two LuxR homologues exist: the AHL binding transcriptional repressor SmaR and the AHL independent transcriptional activator CarR (Cox *et al.*, 1998; Thomson *et al.*, 2000). C4-HSL or C6-HSL binds to the repressor SmaR which is then released and transcription is activated by CarR. The different approaches to regulation of the *car* biosynthetic pathway illustrate how LuxR homologues can act in different ways, possibly as a result of adaptation to different environments and variation in the role within regulatory cascades. In *Serratia* spp., QS is also involved in regulating production of the bioactive pigment prodigiosin (Thomson *et al.*, 2000) and several other interesting phenotypes such as swarming and biofilm formation (reviewed by Van Houdt *et al.* (2007)).

Members of the marine bacterial *Roseobacter* clade are widely isolated from the marine environment, often in association with marine alga (Buchan *et al.*, 2005). Some species of the *Roseobacter* clade are antibacterial (Gram *et al.*, 2010; Hjelm *et al.*, 2004; Martens *et al.*, 2007) and

this is frequently associated to production of the antibacterial compound tropodithietic acid (TDA) (Brinkhoff *et al.*, 2004; Bruhn *et al.*, 2007; Geng *et al.*, 2008; Porsby *et al.*, 2008). AHL production is widespread among *Roseobacter* strains (Bruhn *et al.*, 2007; Gram *et al.*, 2002; Wagner-Döbler *et al.*, 2005) and production of QS signals has been hypothesized to play an important role in *Roseobacter* ecology (Bruhn *et al.*, 2005; Slightom & Buchan, 2009).

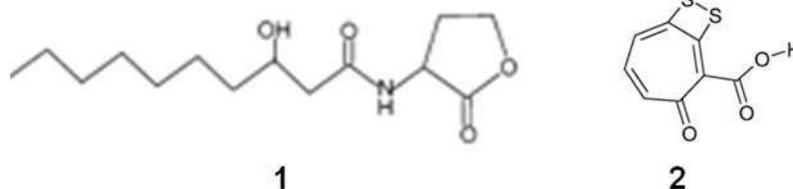


**Figure 22:** QS systems regulating the carbapenem biosynthesis pathway in *Erwinia* and *Serratia* sp. ATCC 39006. In *Erwinia* carbapenem synthesis is induced at high cell density due to the AHL signal binding the LuxR homologue CarR and subsequent induction of the *car* biosynthetic pathway. In contrast, carbapenem biosynthesis in *Serratia* is activated by AHL binding to and inactivating the SmaR repressor, allowing the AHL independent *Serratia* CarR to induce the *car* pathway. Modified from Barnard *et al.* (2007).

The synthesis of TDA is cell-density dependent in some strains (Bruhn *et al.*, 2007), and recent work concluded that production of TDA is under QS control by a 3-OH-C10-HSL induced LuxIR homologue system in *Phaeobacter gallacensis* DSM 17395 (Berger *et al.*, 2011). The *tdaA* gene regulates expression of the TDA biosynthetic pathway and is induced by the LuxR-homologue PgaR. As expected PgaR induces *tdaA* expression when stimulated with the Pgal AHL-product, but curiously TDA can also activate PgaR and induce *tdaA*. Although there is no distinct structural similarity between TDA and the cognate AHL (figure 23), TDA effectively functions as an

autoinducer acting on parts of a LuxIR homologue system, although much higher concentrations of TDA are needed to induce similar expression levels (Berger *et al.*, 2011).

**Figure 23:** Chemical structures of 3-OH-C10-HSL **1**, TDA **2**.



### 4.3 QUORUM SENSING IN *PSEUDOALTEROMONAS*

Only few studies are published on QS in *Pseudoalteromonas*. Two strains have been investigated for QS aspects in their physiology (Guo *et al.*, 2011; Wang *et al.*, 2008), and *Pseudoalteromonas* spp. have been identified as AHL producers in subtidal biofilms (Huang *et al.*, 2008a; Huang *et al.*, 2008b). *Pseudoalteromonas* are also likely included in more general studies of marine microbiota, for instance a survey of coral-associated bacteria revealed *Pseudoalteromonas* strains that activated the biosensors *Agrobacterium tumefaciens* KYC55 or *Escherichia coli* K802NR-pSB1075 (Golberg *et al.*, 2011). *Pseudoalteromonas* sp. 520P1 produces two AHLs, with one inducing production of violacein in the wild type strain (Wang *et al.*, 2008). Recombinant expression of the violacein gene cluster under control of the WT promoter in *E. coli* did, however, not result in violacein production, whereas placing the gene cluster under control of a T7 promoter did (Zhang & Enomoto, 2011). This indicates that a regulatory system native to the WT strain may be essential for expression of the violacein gene cluster from *Pseudoalteromonas* 520P1. However despite attempts to identify *lux* related elements, neither *luxI*, *luxR* or a *lux*-box were identified, thus no conclusive evidence was presented.

AHL synthesis and quorum sensing regulated antibiotic production was reported for *Pseudoalteromonas* sp. NJ6-3-1, related to *P. denitrificans* (BLAST search of reported 16S rRNA nucleotide sequence) (Guo *et al.*, 2011). However, the authors claim that diketopiperazines are the inducer compounds. It is known that diketopiperazines can induce Lux homologue systems,

but it would be nice to see compelling evidence that the native AHLs do not induce a QS response, for instance AHL negative knockouts.

During this Ph.D. work it was discovered that the bioactive strain *P. luteoviolacea* S4054 produces one or more molecules that induce the biosensor strains *Agrobacterium tumefaciens* pZLR4 (Cha *et al.*, 1998) and *Chromobacterium violaceum* CV026 (McClellan *et al.*, 1997) (Vynne *et al.*, 2012c). The *luxI* gene homologue *pluI* was identified through shotgun cloning and expression in *E. coli* JM109-pSB401 (Winson *et al.*, 1998) followed by sequencing by 'primer walking'. Later, whole genome sequencing revealed a putatively QS regulated operon structure downstream of the *pluI* gene, followed by a *luxR* homologue *pluR* (Vynne & Gram, 2012). The genomic organization is shown in figure 24.



**Figure 24:** Genomic organization of QS related elements in *P. luteoviolacea* S4054 and a putatively QS regulated operon. Predicted functions: Dark blue: *pluI*, red: aminotransferase, yellow: dehydrogenase, green: methyltransferase, grey: hypothetical protein, purple: coenzyme F390 synthetase, pink: methylase, brown: lyase, olive: tryptophanyl t-RNA synthetase, light blue: *pluR*. Upstream of *pluI* a palindromic sequence reminiscent of *lux*-boxes was identified (not shown).

The function of the genes downstream of *luxI* is currently unknown, but several of the genes are annotated as enzymes that are known to be active in formation of the antibiotic indolmycin (Hornemann *et al.*, 1971), which correspondingly was identified in strain S4054. In spite of focused efforts it has not been possible to identify the chemical structure of the compound causing activation of the monitor strains. Fractionation using standard approaches in analysis of AHLs (Fekete *et al.*, 2007; Wang *et al.*, 2011) did not result in clear separation of the active compounds into distinct fractions. Raw extracts of the molecules lost the ability to induce a QS response in the biosensors after alkaline treatment but was stable upon acid treatment, as expected for AHLs (Vynne *et al.*, 2012c).

In my studies of a collection of *P. luteoviolacea* strains for phylogenetic and biodiscovery purposes, I discovered that only the indolmycin producing strains synthesized these molecules now denoted

AHL-like (Vynne *et al.*, 2012c). However, all strains produced the purple bioactive pigment violacein which is under QS control in *C. violaceum* (McClellan *et al.*, 1997) and possibly in *Pseudoalteromonas* sp. 520P1 (Wang *et al.*, 2008). In *Serratia*, horizontal transfer of QS related elements have revealed interesting aspects of the impact of QS on microbial physiology. Transfer of a complete prodigiosin biosynthetic cluster to a nonpigmented *Serratia* strain placed prodigiosin biosynthesis under control of the native QS system, and correspondingly, transfer of the *luxIR* homologues *smalR* to a QS negative prodigiosin producing strain placed the native prodigiosin biosynthesis under QS control (Coulthurst *et al.*, 2006). These fascinating experiments show how some biosynthetic elements can be inherently disposed to QS regulation, and may offer an explanation to the variability in QS regulation of violacein synthesis.

#### **4.4 CONCLUSION FROM CHAPTER 4**

Quorum sensing plays an important role in microbial ecology as it enables bacteria to act as coordinated units rather than individual cells. This confers benefits such as biofilm formation, production of defensive antibiotics and expression of virulence factors (Miller & Bassler, 2001; Milton, 2006). Some *Pseudoalteromonas* strains, like many proteobacteria, produce AHLs and possess LuxIR homologue QS machineries which may be involved in regulating production of bioactive compounds. Clearly, the production of antibiotics is quorum sensing regulated in some bacteria and thus QS has a role in microbial interactions both as a direct signal and indirectly through regulating biosynthesis of antagonistic molecules that can affect the microbial community. It is well known that some bacteria have orphan LuxR genes in their genome (Patankar & González, 2009). These may be remnants from previous evolutionary events, but perhaps more interestingly, they may also allow non-AHL producing bacterial cells to “eavesdrop” on conversations among other AHL-producing bacteria. This could allow preemptive production of e.g. antibiotic resistance mechanisms to counter QS-induced antibiotic production or activation of otherwise silenced biosynthetic pathways. Consequently, understanding the role of QS in microbial ecology has the potential to benefit biodiversity efforts focused on novel antibiotics.

## 5.0 CONCLUSION

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### 5.0.1 BIOACTIVE SECONDARY METABOLITES FROM MARINE BACTERIA

This part of the thesis addressed marine bacteria as a source of bioactive secondary metabolites and strategies for biodiscovery efforts. In this study it was confirmed that *Pseudoalteromonas* strains exhibiting stable antibacterial activity are significantly more likely to be isolated from marine surfaces. The potential of marine bacteria to deliver novel bioactive compounds was confirmed. Although no strictly novel antibiotics were discovered, several antibiotics were identified and non-antibiotic novel compounds were discovered. The marine environment has great diversity and, as shown for *P. luteoviolacea*, application of high resolution phylogenetic reconstruction may provide a guiding tool to determine which bacterial strains should be included in further screening efforts. Genome sequencing of the bioactive strain *P. luteoviolacea* S4054 revealed a large unharnessed genetic potential with as many as 11 predicted biosynthetic pathways without a known product. As genome sequencing becomes ever more accessible, whole genome sequencing may become the preferred technique for direct comparison and dereplication of the biosynthetic potential of a collection of marine bacteria. Knowledge of the ecology of the wild type organism is very useful in such a case, as culturing the organism under specific environmentally relevant nutrient levels or physical conditions may result in the production of otherwise absent secondary metabolites, which may not be easily expressed in heterologous hosts.

### 5.0.2 THE GENUS *PSEUDOALTEROMONAS* – ECOLOGY AND BIOACTIVITY

The genus *Pseudoalteromonas* has broad biotechnological applications. This thesis addressed the phylogeny of the genus and its production of antibacterial compounds. The use of secondary metabolites in species identifications was not wholly successful as phylogenetic analysis revealed varying levels of species specificity in secondary metabolite production, with *P. ruthenica* as an example of a narrow species specific secondary metabolite profile and *P. rubra* as an example of a species with a very diverse secondary metabolite profile. The potent antibiotic indolmycin was

discovered in marine bacteria for the first time, which may be evidence of genetic exchange among the terrestrial and marine ecosystems. During the course of the study, a new species was described and given the name *Pseudoalteromonas galathea* after the expedition on which it was first isolated. Within *Pseudoalteromonas luteoviolacea*, three antibiotics were produced in a phylotype specific manner. This provided evidence that these antibiotics are maintained under selective pressure and thus likely are important in microbial ecology.

### **5.0.3 QUORUM SENSING IN REGULATION OF BACTERIAL PRODUCTION OF ANTIMICROBIAL COMPOUNDS**

Quorum sensing regulates a range of potential phenotypes. In the context of this thesis, the interest has primarily been regulation of antibiotic production. A potentially novel AHL-like molecule was described from *P. luteoviolacea* S4054, and this may take part in regulation of a novel biosynthetic pathway. However, such molecules may also be involved in countless other interactions when released into a microbial community. Understanding novel quorum sensing systems and their influence on microbial interactions could help us not only discover novel natural products, but also to better understand the regulatory events in complex microbial communities both in nature and in medicine.

## 5.1 OUTLOOK

Discovery of novel antibiotics and other strategies to combat the spread of resistant human pathogenic bacteria will remain a priority. Efficient screening strategies combined with sampling of novel microbial diversity will likely remain one of the most promising avenues to achieve this, although revisiting known antibiotic producing strains with modern techniques may be necessary.

In the near future, whole genome sequencing and metagenome approaches appear poised to dominate within natural product discovery. This will offer nearly unlimited access to genetic and chemical diversity, and allow dereplication of biosynthetic gene clusters as one of the first steps in a biodiscovery program. As a result, the discovery of molecules with coveted pharmaceutical properties will accelerate. In addition, selective targeting of specific microbial communities for sequencing, e.g. sponge symbiotic bacteria or bacteria from dense biofilms, may lead to a higher rate of discovery than otherwise obtained. The use of heterologous expression systems will provide access to some of these compounds, yet some gene clusters may not be properly expressed, or the end product may be toxic to the producer cell. Alternatively, combining genomic approaches with knowledge of the ecology and physiology of a bioactive bacterium may lead to production of novel compounds by the wild type strain. Genome sequencing would reveal the presence of any known signaling systems, which could then be activated to potentially induce novel biosynthetic pathways, or growth conditions may be adjusted based on *in silico* reconstructions of the metabolic network of the organism in order to induce expression of biosynthetic clusters, for instance by culturing under simulated natural conditions.

Knowledge of *in situ* microbial interactions and the role antibiotics play in the microbial community will naturally be of interest to microbial ecologists, but it may also help us understand why important phenomena such as resistance to antibiotics evolve, adapt and persist in the environment, and potentially make us better equipped to counter the emergence of antibiotic resistance in pathogenic bacteria.

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## PAPER 1

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Vynne N, Månsson M, Nielsen KF, Gram L (2011)

Bioactivity, chemical profiling, and 16S rRNA-based phylogeny of *Pseudoalteromonas* strains collected on a global research cruise.

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# Bioactivity, Chemical Profiling, and 16S rRNA-Based Phylogeny of *Pseudoalteromonas* Strains Collected on a Global Research Cruise

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**Abstract** One hundred one antibacterial *Pseudoalteromonas* strains that inhibited growth of a *Vibrio anguillarum* test strain were collected on a global research cruise (Galathea 3), and 51 of the strains repeatedly demonstrated antibacterial activity. Here, we profile secondary metabolites of these strains to determine if particular compounds serve as strain or species markers and to determine if the secondary metabolite profile of one strain represents the bioactivity of the entire species. 16S rRNA gene similarity divided the strains into two primary groups: One group (51 strains) consisted of bacteria which retained antibacterial activity, 48 of which were pigmented, and another group (50 strains) of bacteria which lost antibacterial activity upon sub-culturing, two of which were pigmented. The group that retained antibacterial activity consisted of six clusters in which strains were identified as *Pseudoalteromonas luteoviolacea*, *Pseudoalteromonas aurantia*, *Pseudoalteromonas phenolica*, *Pseudoalteromonas ruthenica*, *Pseudoalteromonas rubra*, and *Pseudoalteromonas piscicida*. HPLC-UV/VIS analyses identified key peaks, such as violacein in *P. luteoviolacea*. Some compounds, such as a novel bromoalterochromide,

were detected in several species. HPLC-UV/VIS detected systematic intra-species differences for some groups, and testing several strains of a species was required to determine these differences. The majority of non-antibacterial, non-pigmented strains were identified as *Pseudoalteromonas agarivorans*, and HPLC-UV/VIS did not further differentiate this group. *Pseudoalteromonas* retaining antibacterial were more likely to originate from biotic or abiotic surfaces in contrast to planktonic strains. Hence, the pigmented, antibacterial *Pseudoalteromonas* have a niche specificity, and sampling from marine biofilm environments is a strategy for isolating novel marine bacteria that produce antibacterial compounds.

**Keywords** *Pseudoalteromonas* · Antibacterial activity · Secondary metabolites · Bioprospecting · Galathea 3

## Introduction

Compounds of relevance for the pharmaceutical and biotechnology industries are produced by marine microorganisms (Burgess et al. 1999), and it has been suggested that some compounds of pharmacological interest previously attributed to macroorganisms may in fact be of microbial origin (Bewley and Faulkner 1998; Simmons et al. 2008; Sudek et al. 2006). The emergence of multiresistant pathogenic bacterial strains and the failure of combinatorial and diversity-oriented chemistry to adequately supply the drug discovery pipeline (Newman 2008) have re-invigorated natural product chemistry as a path for discovery and development of new antibiotics. With this in mind, we isolated marine bacteria with antibacterial activity during the Danish Galathea 3 marine research expedition (Gram et al. 2010). The antibacterial strains were tentatively identified using 16S rRNA similarity, and one of the major groups of

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isolated bacteria was identified as *Pseudoalteromonas* (Gram et al. 2010).

The genus *Pseudoalteromonas* consists of Gram-negative marine bacteria belonging to the  $\gamma$ -proteobacteria and is present globally in marine waters where they constitute 0.5% to 6% of the total bacterioplankton (Wietz et al. 2010). They are heterotrophic aerobes and non-fermentative, and the cells are motile by one or more polar flagella. The genus divides into two groups: pigmented and non-pigmented species. The pigmented species are often producers of bioactive secondary metabolites (Bowman 2007) displaying cytotoxic (Zheng et al. 2006), antibacterial (Gauthier 1976b; Gauthier and Flatau 1976; Isnansetyo and Kamei 2003; Jiang et al. 2000; McCarthy et al. 1994), antifungal (Franks et al. 2006; Kalinovskaya et al. 2004), or antifouling (Egan et al. 2001; Holmström et al. 2002) effects. It has been hypothesized that bioactive *Pseudoalteromonas* are primarily associated with higher organisms (Holmström and Kjelleberg 1999), suggesting an ecological role in which some bioactive species might play an active part in host defense against pathogens and fouling organisms (Holmström et al. 1996; Armstrong et al. 2001; Egan et al. 2008). A link between surface colonization and antibacterial activity has not been experimentally verified, although several studies have successfully isolated epiphytic bacteria with antibacterial activity from algae and other marine organisms (Armstrong et al. 2001; Boyd et al. 1999; James et al. 1996; Penesyan et al. 2009). The group of non-pigmented species has highly similar 16S rRNA gene sequences (Ivanova et al. 2004) and is rarely inhibitory against other microorganisms, although, *Pseudoalteromonas haloplanktis* strain INH produces isovaleric acid and 2-methylbutyric acid showing a broad spectrum of bacterial inhibition (Hayashida-Soiza et al. 2008).

Phylogeny and differentiation of bacterial species rely heavily on 16S rRNA gene similarity (Stackebrandt et al. 2002); however, 16S rRNA gene similarity does not provide sufficient differentiation below species level (Fox et al. 1992). Comparison of secondary metabolite production has supported species delineation within the actinomycete genus *Salinispora*, where strains with high 16S rRNA similarity were shown to belong to distinct species each with different specific metabolite profiles (Jensen et al. 2007). In mycology, comparison of chemical profiles (e.g., TLC, direct-infusion mass spectrometry, and HPLC with various detectors such as UV/VIS and/or mass spectrometry) of secondary metabolites has been widely used to identify and differentiate filamentous fungi (chemotaxonomy) also at sub-species level (Frisvad et al. 2008), and the chemophylogeny correlates well with phylogenetic analysis of sequences of specific housekeeping genes (e.g., chitin synthase,  $\beta$ -tubulin, and calmodulin; Geiser et al. 2007). Since several *Pseudoalteromonas* species produce a range of

secondary metabolites, we hypothesized that chemical profiling and specific marker compounds could be indicative of bioactive potential and at the same time be useful in species identification or differentiation (Jensen et al. 2007).

The aim of the present study was to profile the secondary metabolites of these strains to determine if particular compounds serve as markers of strains or species with antibacterial activity and to determine if several strains of each species must be tested to assess the full bioactivity potential. As part of this, we provide accurate identification and phylogeny of these organisms by detailed 16S rRNA gene sequence comparative analysis. Since the bacteria were isolated from different sample types, our collection also allows us to address aspects of *Pseudoalteromonas* ecology such as the possible link between surface or planktonic lifestyle and antibacterial activity.

## Materials and Methods

**Strain Isolation** Approximately 500 marine bacterial strains with antagonist activity against *Vibrio anguillarum* strain 90-11-287 (Skov et al. 1995) were isolated during the Danish Galathea 3 research expedition (Gram et al. 2010). One hundred one of these strains tentatively identified as *Pseudoalteromonas* species were included in the present study. *V. anguillarum* 90-11-287 was used as target strain since the expedition ship was not equipped to handle, e.g., potential human pathogens, and this *Vibrio* strain is in our experience very sensitive to antibacterial compounds from other marine bacteria (Hjelm et al. 2004).

**Growth Media and Culture Conditions** *Pseudoalteromonas* strains were grown in marine broth (MB) 2216 (Difco, Detroit, MI, USA) and on marine agar (MA) 2216 (Difco, Detroit, MI, USA) prepared in accordance with the manufacturer's instructions. Broth cultures were incubated under stagnant conditions at 25°C. Pigment production was determined by visual inspection of 48-h-old culture broths (MB) and colonies grown on MA for 24 to 48 h.

**Antibacterial Activity** Instant Ocean (IO) bioassay agar plates were prepared as described by Hjelm et al. (2004). Ten grams per liter agar, 3.3 g/l casamino acids (Difco 223050, Detroit, MI, USA), and 30 g/l Instant Ocean aquatic salts (Instant Ocean® Aquarium systems Inc., Sarrebourg, France) were added to distilled water and autoclaved. Glucose (0.4%) and 10  $\mu$ l/ml of *V. anguillarum* overnight culture were added to the cooled (44°C) IO and plates poured. The plates were allowed to dry for 15 min, and if used for well diffusion agar assays (WDAA), wells (diameter 6 mm) were punched. The inhibitory activity of live *Pseudoalteromonas* bacterial cells was tested by

spotting 48-h-old MA grown colonies on freshly prepared IO agar plates containing *V. anguillarum*. Plates were incubated at 25°C and inspected for clearing zones in the growth of *V. anguillarum* after 24 h.

Cell-free supernatants were prepared to test for the presence of water-soluble antibacterial compounds secreted to the broth, and ethyl acetate extracts were prepared to test for production of non-polar antibacterial compounds. Each strain was grown in 20 ml of MB for 48 h. A 1.5-ml sample was withdrawn for 0.2 µm filtering, and subsequently the remainder of the culture was extracted with an equal volume of ethyl acetate. The ethyl acetate fraction was transferred to a new vessel, evaporated to dryness, and redissolved in 2×0.5 ml ethyl acetate. The 1.5-ml cell-free sterile-filtered supernatant and the ethyl acetate extracts were stored at –20°C until tested in the WDAA (50 µl sample per well) based on IO agar plates containing *V. anguillarum*. Controls (sterile MB and pure ethyl acetate) did not cause any inhibition zones.

The number of antibacterial *Pseudoalteromonas* strains in surface samples (e.g., algae, driftwood, fish, and sediment samples) was compared to their numbers in water samples by the Fisher's exact test (Fisher 1958). A 2×2 contingency table was used to test the hypothesis that presumed antibacterial strains with stable antibacterial activity were equally likely to be isolated from water samples and surface samples.

**16S rRNA Gene Sequence Analyses** A detailed phylogenetic analysis was performed on 16S rRNA sequences obtained in a previous study (Gram et al. 2010). For the analysis in this study, we conducted a BLAST (<http://blast.ncbi.nlm.nih.gov>) search against a compilation of *Pseudoalteromonas* type strain sequences retrieved from GenBank (list of type strains obtained from <http://www.bacterio.cict.fr>), and sequences of the type strains with a BLAST match in our strain collection were included in 16S rRNA sequence analysis to obtain a robust phylogenetic tree. Sequences from two additional *Pseudoalteromonas* strains were included: The genus type strain *P. haloplanktis* and the bioactive *Pseudoalteromonas tunicata*. *Salinispora arenicola* CNS-205 was used as outgroup. The sequences were aligned by the MAFFT online software (<http://www.ebi.ac.uk/Tools/mafft/>; Katoh et al. 2002) and curated with the Gblocks software on its least stringent settings (Castresana 2000; Talavera and Castresana 2007). The resulting alignment was processed using the MEGA4 software (Tamura et al. 2007) to create neighbor-joining and minimum evolution trees. PhyML 3.0 was used to generate a maximum likelihood tree (Guindon and Gascuel 2003). Phylogenetic trees were generated under default parameters with 1,000 bootstrap replications for neighbor-joining and minimum evolution trees and 100 bootstrap replications for the maximum likelihood tree.

GenBank accession numbers for the *Pseudoalteromonas* strains used in this study are included in Supplementary Table 1.

**HPLC-UV/VIS Analysis of Secondary Metabolites** The strains were grown in static cultures in 10 ml MB for 3 days at 25°C, and for each species, one strain was cultured in triplicate to establish extraction and growth variation. Cultures were extracted with equal volumes of ethyl acetate, centrifuged, and the ethyl acetate was evaporated under N<sub>2</sub> to dryness. Samples were redissolved in 300 µl acetonitrile–water (1:1 v/v) and filtered through a 13-mm ID PTFE syringe filter. A subsample of 2 µl was then analyzed by reversed phase HPLC on an Agilent 1100 System equipped with a UV/VIS photo diode array detector (scanning 200–600 nm). Separation was done on a 100 mm×2 mm i.d., 3 µm Gemini C<sub>6</sub>-phenyl column (Phenomenex, Torrance, CA, USA), running at 40°C using a binary linear solvent system of water (A) and acetonitrile (B; both buffered with 50 µl/l trifluoroacetic acid) at a flow of 300 µl/min. The gradient profile was *t*=0 min, 5% B; *t*=22 min, 70% B; *t*=24.5, 100% B; *t*=27 min, 100% B; and *t*=29 min, B=5%, holding this for 8 min prior to the next injection. The chromatographic profiles were compared, subtracting peaks present in media blank extracts. Samples were analyzed in random order, and six of the first extracts were analyzed several times during the sequence to determine any retention time shifts. Cluster analysis was done on a matrix of detected / non-detected peaks (1/0) using NTSYSpc 2.20q (Exeter Software, Setauket, NY, USA). SAHN clustering was used by unweighted pair-group method (UPGMA) and simple distance measurement. Representative extracts were also analyzed by HPLC-UV/VIS-TOFMS in both positive and negative electrospray (Nielsen and Smedsgaard 2003). Peaks were tentatively identified by UV spectra and accurate mass data by matching in Antibase 2009 (35 930 microbial secondary metabolites; Wiley & Sons, Hoboken, NJ, USA; Nielsen et al. 2006).

## Results

**Pigmentation and Antibacterial Activity** The one hundred one *Pseudoalteromonas* strains were originally isolated for their ability to inhibit *V. anguillarum* (Gram et al. 2010). However, on re-cultivation and re-testing for the ability to inhibit *V. anguillarum* after storage at –80°C for several months, only 51 strains retained inhibitory activity (Table 1). These 51 strains were all inhibitory when tested as live cultures in the “spot test” assay. Twenty of the strains produced water-soluble, diffusible, antibacterial substances as indicated by the ability of cell-free sterile-filtered supernatant to inhibit growth of *V. anguillarum* in the

**Table 1** Identity, antibacterial activity, and pigmentation of *Pseudoalteromonas* strains from a global collection

16S rRNA cluster	No. of strains		Related type strain <sup>a</sup>	No. of strains from		No. of strains inhibiting <i>Vibrio</i>		Inhibition of <i>Vibrio</i> by EtAc extracts
	Pigmented	Non-pigmented		Surface samples	Water samples	Pigmented	Non-pigmented	
I	1	37	<i>P. agarivorans</i>	16	22	0	0	0
II	5	0	<i>P. aurantia</i>	2	3	5	0	0
III	0	9	<i>P. prydzensis</i>	8	1	0	0	0
IV	3	3	<i>P. phenolica</i>	3	3	3	2	0
V	4	0	<i>P. luteoviolacea</i>	4	0	4	0	4
VI	9	0	<i>P. rubra</i>	9	0	9	0	0
VII	13	0	<i>P. flavipulchra</i>	13	0	13	0	0
VIII	15	0	<i>P. ruthenica</i>	14	1	15	0	15
S1727	0	1	<i>P. mariniglutinosa</i>	0	1	0	0	0
S3655	1	0	<i>P. spongiae</i>	1	0	0	0	0
Total	51	50		70	31	49	2	19

<sup>a</sup> The type strain which the majority of the strains in the cluster were most closely related to

WDAAs. These 20 strains were identified as *Pseudoalteromonas phenolica* (one strain), *Pseudoalteromonas luteoviolacea* (five strains), *Pseudoalteromonas rubra* (nine strains), *Pseudoalteromonas citrea* (one strain), and *Pseudoalteromonas aurantia* (four strains). Ethyl acetate extraction of culture broths resulted in 19 crude extracts which inhibited growth of *V. anguillarum* in the WDAAs. Four of these were identified as *P. luteoviolacea* and were the only strains where both cell-free supernatant and crude ethyl acetate extracts inhibited *Vibrio* growth. The remaining 15 inhibitory crude extracts all originated from strains identified as *Pseudoalteromonas ruthenica*. The crude ethyl acetate extracts of 26 strains were intensely colored; however, only some of these extracts inhibited growth of *Vibrio* indicating that the pigments were not universally antibacterial. Cell-free culture supernatants and ethyl acetate crude extracts of strains with no growth inhibition of *V. anguillarum* in the “spot test” assay were also tested but showed no growth inhibition.

Forty-eight of the 51 antibacterial strains were pigmented, while two (S3431 and S3655) of the 50 non-active strains were pigmented (Table 1). Antibacterial activity was significantly more likely to be produced by pigmented strains as determined by Fisher’s exact test (two-tailed *p* value of 0.0000). In total, 70 strains were isolated from surface swabs and 31 from water samples. Of the surface-associated strains, 45 remained active in the spot-assay in comparison to six of the water sample strains. The Fisher’s exact test demonstrated a significant relation between surface association and stable antibacterial activity (two-tailed *p* value of 0.0000).

*Pseudoalteromonas* strains were isolated on all parts of the global cruise in both tropical and temperate waters (Fig. 1). Our strain collection is not large enough to allow

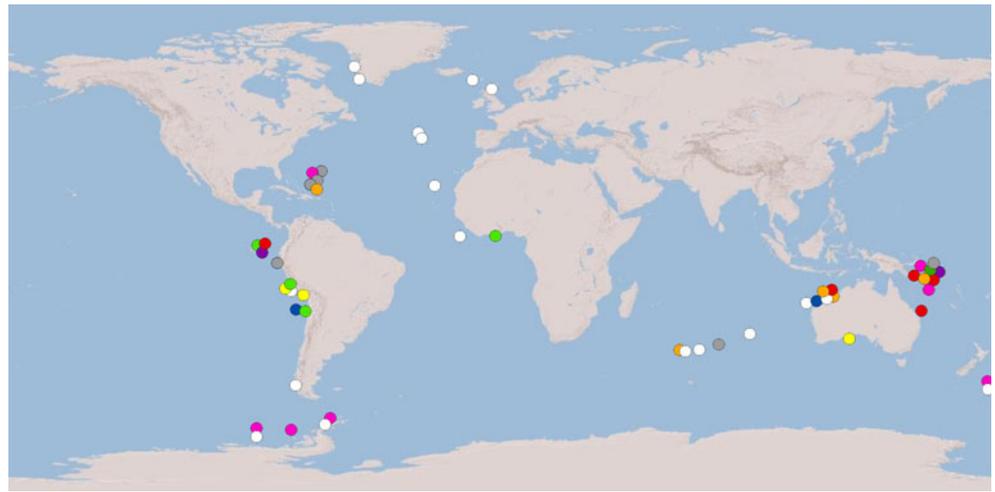
for complete biogeographic analysis, but pigmented strains appeared to be more frequent in coastal areas whereas the non-pigmented strains appeared associated with open waters (Fig. 1).

**16S rRNA Gene Sequence Analyses** We initially performed a BLAST search querying the 16S rRNA gene sequence of each strain against the GenBank database. The results of this analysis were ambiguous, as some sequences returned more than 40 hits all with identical scores in the BLAST results (data not shown), frequently including the sequences of several different *Pseudoalteromonas* species. Therefore, a BLAST analysis was carried out querying the sequences against a complete set of *Pseudoalteromonas* type strains, and hence, each strain is matched with the best type strain BLAST match (Suppl Table 1).

The 16S rRNA gene sequences were used to cluster the strains by constructing a neighbor-joining tree, and branch support was verified by comparison to minimum evolution and maximum likelihood trees (Fig. 2). Nodes supported by an 80% bootstrap cutoff were collapsed when three or more strains were included in the cluster (Fig. 2). An exception was made for clusters VI and VII, which are shown as separate clusters due to obvious differences in phenotype (pigment, bioactivity, secondary metabolite profile). Ninety-nine of the strains fell into one of eight primary clusters.

Clusters I and III consisted of non-pigmented non-inhibitory strains (Table 1). Cluster I included 38 strains and the type strains of *P. haloplanktis*, *Pseudoalteromonas agarivorans*, *Pseudoalteromonas tetraodonis*, *Pseudoalteromonas paragorgicola*, *Pseudoalteromonas distincta*, *Pseudoalteromonas arctica*, *Pseudoalteromonas nigrifaciens*, *Pseudoalteromonas*

**Fig. 1** Isolation sites of 101 *Pseudoalteromonas* strains with each color code representing one of the eight clusters as indicated in Table 1. Each circle represents the isolation of one or more strains belonging to the given cluster. White=cluster I, yellow=cluster II, gray=cluster III, green=cluster IV, violet=cluster V, red=cluster VI, orange=cluster VII, and pink=non-clustered strain

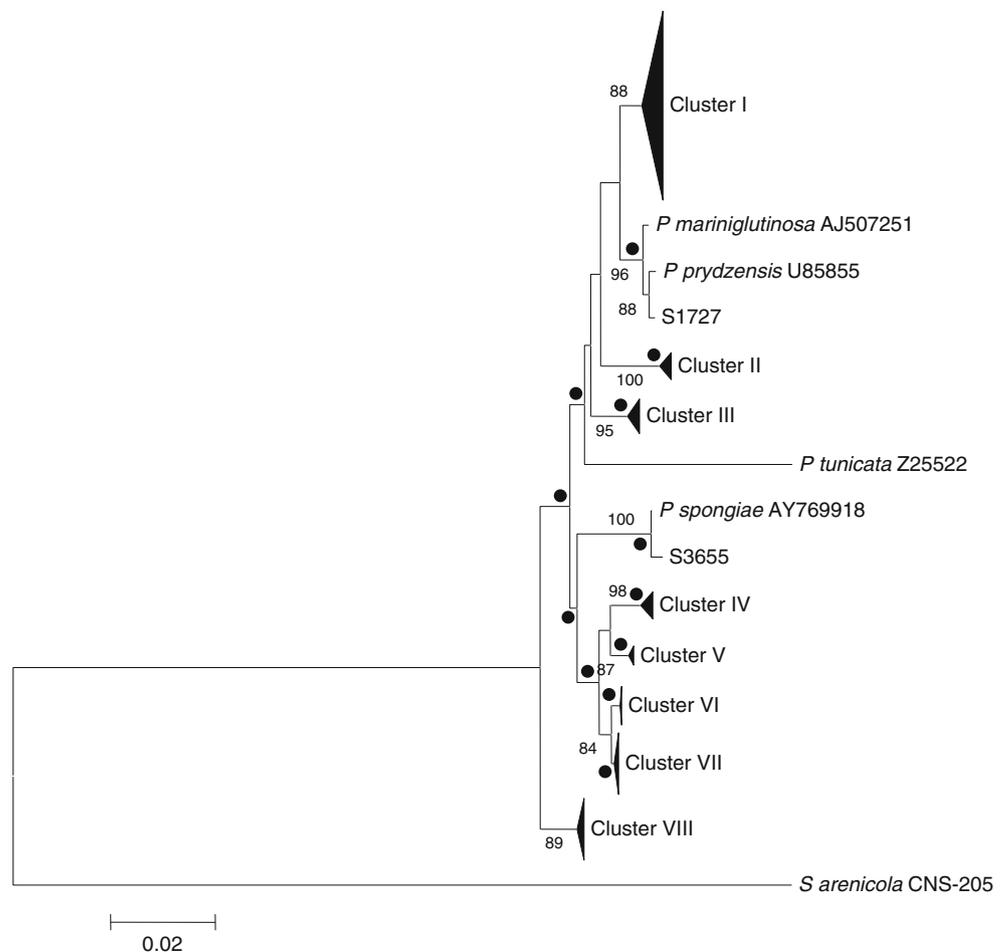


*elyakovii*, *Pseudoalteromonas carrageenovora*, *Pseudoalteromonas marina*, and *Pseudoalteromonas aliena*. Strain S3431—a black-pigmented strain in cluster I—did not show more than 97% similarity even when compared to the full GenBank database which suggests that S3431 could represent a novel *Pseudoalteromonas* species. Despite the low BLAST similarity score, phylogenetic analysis and tree construction

placed strain S3431 in the diverse cluster I (the non-collapsed cluster I is shown in Supplementary Figure 1). Cluster III contained no type strains which supported the BLAST analysis where the strains in cluster III were 98% similar to the best type strain match (Supplementary Table 1).

The remaining six of the eight clusters contained pigmented strains. Pale yellow strains clustered with the

**Fig. 2** Phylogenetic tree based on 16S rRNA gene sequences of the Galathea 3 *Pseudoalteromonas* strains. Sequences were aligned by MAFFT (default options), and the resulting alignment was used to generate a neighbor-joining tree in the MEGA4 software package (default settings, 1,000 bootstraps). *S. arenicola* CNS-205, GenBank accession number CP000850, GeneID: 5705939 was used as outgroup. Clusters containing two or more non-type strain sequences were collapsed. The scale bar represents 0.02 amino acid substitutions per nucleotide position. Circle nodes also occurred in minimum evolution and maximum likelihood trees



type strains of *P. citrea* and *P. aurantia* (cluster II) and four intensely purple strains grouped in cluster V with the type strain of *P. luteoviolacea*. Cluster VI contained nine red-pigmented strains and the type strain of *P. rubra*, and cluster VII consisted of 12 intensely yellow strains, one pale yellow strain, and the *Pseudoalteromonas flavipulchra*, *Pseudoalteromonas maricaloris*, and *Pseudoalteromonas piscicida* type strains. Fifteen strains and their nearest BLAST match, *P. ruthenica*, formed cluster VIII. These strains all produced a pale brown pigment. Cluster IV contained six strains and the type strain of *P. phenolica*. Four of the strains in this cluster had *P. phenolica* as their best type strain BLAST match; however, strain S1093 had *P. luteoviolacea* as its best match at 98% identity, while *P. rubra* and *P. luteoviolacea* type strains scored identically (97%) as the best matches for S2724. The strains in cluster IV were heterogeneous with respect to pigmentation, some were non-pigmented and others appeared brown.

**Profiling of Secondary Metabolites** The 101 strains and select type strains were separable in discrete groups by HPLC-UV/VIS (Fig. 3), and the triplicate profiles from an isolate of each species were very reproducible and could be superimposed on each other (data not shown). All of the 38 strains of the 16S rRNA cluster I fell into group A, in which no UV/VIS peaks were unique compared to the media blanks indicating that no secondary metabolites were produced. This large group also included all nine strains from 16S rRNA cluster III and strains of *P. phenolica* and *P. ruthenica* less proficient in secondary metabolite production. A summary of the detected compounds is shown in Table 2, and the producer organisms are shown by 16S rRNA cluster in Table 3.

Based on their production of specific metabolites, the majority of pigmented bacteria formed six main groups not including four *P. rubra* strains (Fig. 3). In the pigmented bacteria, a total of 26 distinct peaks were detected and included in the cluster analysis. We identified indolmycin, violacein, and prodigiosin among the significant peaks based on reference standards. Furthermore, nine peaks could be tentatively identified based on HPLC-UV/VIS-TOFMS results and data in Antibase2009. These nine included two likely novel bromoalterochromides and a brominated indole (Table 2).

Comparing the 16S rRNA gene sequence clusters with the chemical profiling revealed several patterns. Some compounds were exclusively produced by strains belonging to the same cluster whereas other compounds were produced across several strains from different clusters. All strains in cluster II (*P. aurantia*/*P. citrea*) shared production of compound B (retention time, RT 12.31 min) whereas the production of four other compounds F, Q, T, and U (RT 15.47, 16.60, 17.96, and 18.28 min) were scattered in the

group. This included a novel bromoalterochromide (compound Q, RT 16.60 min) that was also found in cluster VII (*P. flavipulchra*/*P. piscicida*). Three of the other compounds were identified as quinolines based on distinct UV spectra and accurate mass.

*P. luteoviolacea* strains (cluster V) shared production of compound D (violacein, RT 14.29) in all four strains and the type strain but were sub-divided by compound A (indolmycin, RT 11.21) produced by two strains and compound Z (pentabromopseudilin, RT 22.65) produced by the two other strains and the type strain. This division is visible in Fig. 4, which shows chromatograms of the four strains in cluster V. Interestingly, also two *P. phenolica* strains produced compound Z.

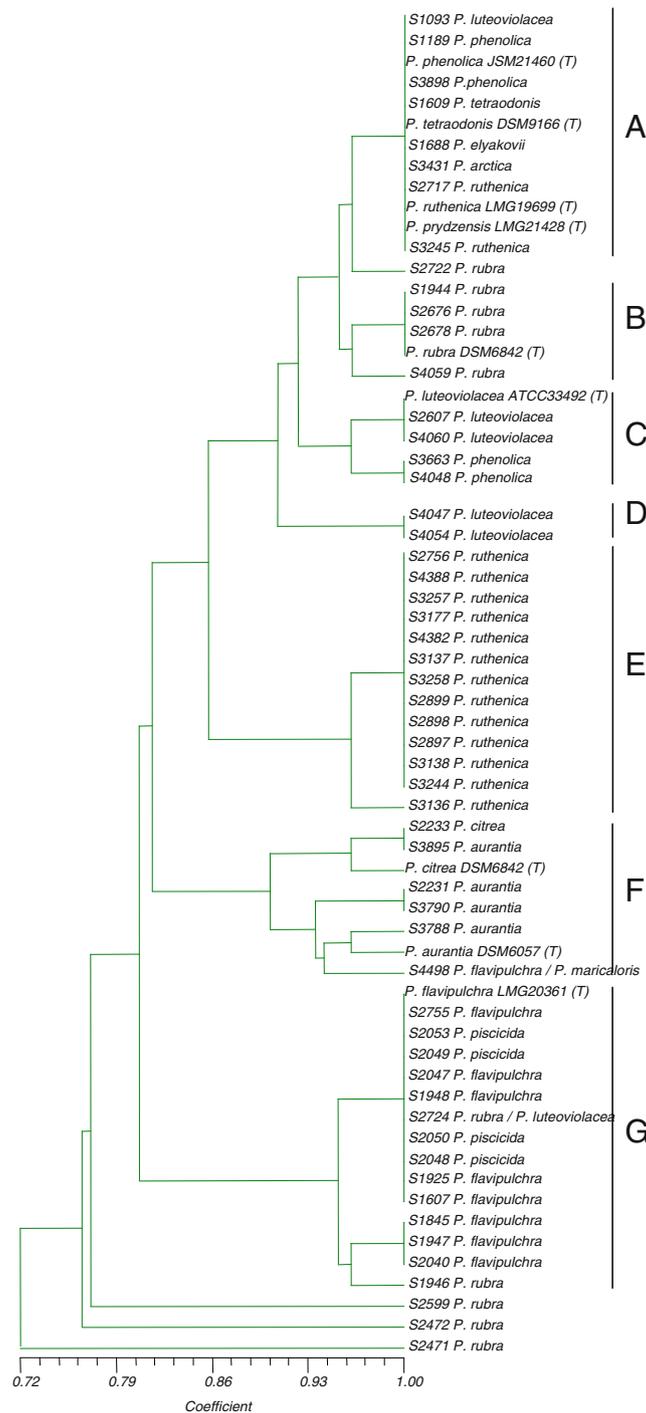
Cluster VII (*P. flavipulchra* and *P. piscicida*) was chemically very homogeneous. All strains except one produced three bromoalterochromides P, Q, and R (RT 16.49, 16.60, and 17.10) of which Q and R were novel compounds. In contrast to cluster V and compound D, the production of P, Q, and R was not a unique marker for strains of this cluster as P, Q, and R were also detected in one strain from cluster II and one strain from cluster VI.

Thirteen of the 15 strains in cluster VIII, identified as *P. ruthenica*, shared a unique chemical profile and produced the compounds H, K, and O (RT 15.78, 15.98, 16.30) with characteristic UV spectra. None of these matched compounds in Antibase2009 and potentially constitute novel antibacterials. These compounds were not detected among strains from other clusters, yet they were not suitable as a distinct chemical marker for cluster VIII since no secondary metabolites were detected in the remaining two strains in this cluster or the type strain.

The strains in cluster VI were identified as *P. rubra* and were chemically very heterogeneous. Five of nine strains produced the red pigment prodigiosin (compound M, RT 16.00) which was not detected in strains of other clusters. Additionally, a multitude of known and non-identified compounds were detected in one or more strains in the cluster. In total, 16 compounds were detected within the cluster, and 12 of these were unique for this cluster. Only two strains shared an identical production of secondary metabolites, further stressing the chemical diversity among the strains in this cluster.

## Discussion

We demonstrate in this study, in agreement with earlier findings (Bowman 2007), that species within the *Pseudoalteromonas* genus produce a range of secondary metabolites, some with antibacterial activity. Several species of the genus are intensely pigmented, and it is



**Fig. 3** Dendrogram from cluster analysis of detected peaks from HPLC-UV/VIS detection of compounds in the ethyl acetate extracted broth and biomass. Data were processed in NTSYSpc 2.20q, with SAHN clustering by UPGMA and simple distance measurement

hypothesized that pigmentation co-occur with antibacterial activity (Egan et al. 2002). In our global collection of *Pseudoalteromonas* strains that demonstrated antibacterial activity on initial isolation, strains that were pigmented were significantly more likely to retain antibacterial activity on re-growth than non-pigmented strains. Several

intensely colored organic extracts were not inhibitory against *V. anguillarum*, and hence, we do not believe that the pigments, in general, are the cause of the antibacterial activity although, e.g., the purple pigment violacein is a known antibiotic compound (Lichstein and Vandesand 1945).

**Table 2** Ethyl acetate extractable secondary metabolites produced by pigmented *Pseudoalteromonas* strains

Compound	MI (Da) <sup>c</sup>	UV-max data	RT (min)	Code
Indolmycin <sup>a</sup>	257		11.21	A
2-Pentyl-4-quinolinol <sup>b</sup>	215		12.31	B
Novel mono-brominated indole	280		13.78	C
Violacein <sup>a</sup>	343		14.29	D
Unidentified	244	212 nm (100%), 250 nm (48%)	15.21	E
2- <i>n</i> -Heptyl-(1 <i>H</i> )-quinolin-4-one <sup>b</sup>	243		15.47	F
Unidentified	NI	228 nm (45%), 308 nm (100%)	15.70	G
Unidentified	386	<200 nm	15.78	H
Unidentified	316	286 nm (100%)	15.80	I
Unidentified	NI	<200 nm	15.81	J
Unidentified	NI	228 nm (45%), 308 nm (100%)	15.98	K
Unidentified	676	<200 nm	15.99	L
Prodigiosin <sup>a</sup>	323		16.00	M
Unidentified	NI	<200 nm	16.26	N
Unidentified	NI	228 nm (45%), 308 nm (100%)	16.30	O
Bromoalterochromide A <sup>b</sup>	843		16.49	P
Novel bromoalterochromide, 2 bromine	921		16.60	Q
Novel bromoalterochromide, 1 bromine	857		17.10	R
Unidentified	333	310 nm (100%)	17.20	S
2- <i>n</i> -Nonyl-(1 <i>H</i> )-quinolin-4-one <sup>b</sup>	271		17.96	T
Nonyl-quinolinone analog <sup>b</sup>	271		18.28	U
Unidentified	315	362 nm (100%)	18.90	V
Unidentified	244	218 nm (100%), 280 nm (82%)	19.42	W
Unidentified	NI	218 nm (100%), 288 nm (82%)	19.78	X
Unidentified	NI	250 nm (100), 280 nm (86)	19.92	Y
Pentabromopseudilin <sup>b</sup>	549		22.65	Z

NI no ionization or MI could not be assigned using ESI<sup>+</sup> and ESI<sup>-</sup>

<sup>a</sup> Validated reference standard used for verification

<sup>b</sup> Accurate mass and UV data fit the data from Antibase2009

<sup>c</sup> Mono-isotopic mass

Nearly half of the isolated strains did not retain any antibacterial effect after frozen storage and sub-culturing despite being isolated on the original plates due to antibacterial activity. The observed loss of antibacterial activity may be due to a requirement for factors specific to local seawater, as initial tests for antibacterial activity were carried out using 50% local seawater (Gram et al. 2010). Furthermore, loss of antibacterial activity may be due to repression or inhibition of gene clusters encoding products that are required for secondary metabolite synthesis (e.g., by catabolite repression). A reduction in antibiotic production when the producer organism is grown in excess of a carbon source is a known phenomenon (Sanchez et al. 2010), and suppression of secondary metabolite production by excess concentrations of other substrate components is demonstrated in *Streptomyces* (Doull and Vining 1990). This could suggest

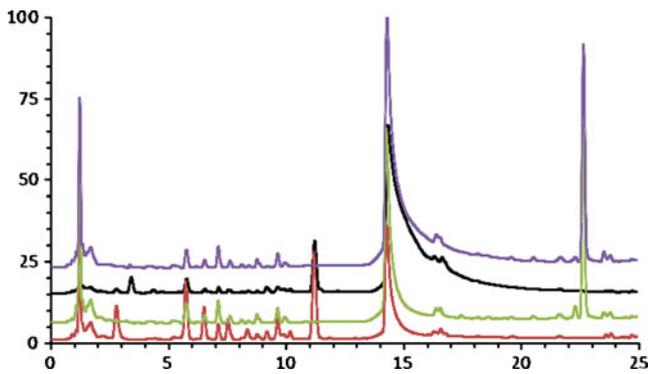
that culturing the strains under nutrient limited conditions may reestablish production of antibacterial compounds. Also, during the original sampling and screening procedure, the agar plates may have harbored co-cultured microorganisms which potentially induce antibacterial activity as has been demonstrated by Meams-Spragg et al. (1998). Hence, it may be possible to re-induce the antibacterial activity if the right conditions can be created.

Several bioactive *Pseudoalteromonas* have been isolated from higher organisms, and it has been hypothesized that antibacterial compounds may play a role in bacterial competition or as protective agents beneficial for the host organism (Holmström and Kjelleberg 1999). We provide statistical evidence that surface-associated presumed antibacterial pseudoalteromonads are significantly more likely to show stable production of antibacterial compounds than

**Table 3** Secondary metabolites produced by *Pseudoalteromonas* species clustered by 16S rRNA gene similarity

16S cluster	# strains	Peak at retention time present in <i>Pseudoalteromonas</i> strain/organism																											
		None	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	
I	38	x																											
<i>P. tetradonis</i>	DSM9166	x																											
<i>P. prydzensis</i>	LMG21428	x																											
II	1		x				x														x								
	2		x				x														x								
	1		x				x														x								
	1		x				x																						
<i>P. aurantia</i>	DSM6057		x																		x								
<i>P. citrea</i>	DSM8771		x																										
III	9	x																											
IV	3	x																											
	1																												
	2																												
<i>P. phenolica</i>	DSM21460	x																											
V	2						x																						
	2						x																						
<i>P. luteoviolacea</i>	ATCC33492		x																										
VI	1																												
	1																												
	1																												
	1																												
	1																												
<i>P. rubra</i>	DSM6842																												
VII	9																												
	3																												
	1																												
	1																												
	1																												
<i>P. flavipulchra</i>	LMG20361																												
VIII	2																												
	13																												
<i>P. ruthenica</i>	LMG19699	x																											
S1727	1	x																											
S3655	1	x																											

Identification of peak by capital letter in Table 2



**Fig. 4** HPLC-UV/VIS profiles of the ethyl acetate extracted broth and biomass of four *P. luteoviolacea* with the tailing violacein peak at 14–15 min and in two traces pentabromopseudilin at 22–23 min in contrast to indolmycin at 11 min in the two other traces

*Pseudoalteromonas* species isolated as planktonic cells. This suggests that production of antibacterial compounds may play an important role in the ability of *Pseudoalteromonas* strains to colonize and persist on surfaces submerged in the marine environment, as previously suggested for *P. tunicata* strain D2 (Rao et al. 2005).

The analysis of 16S rRNA gene sequences from our global collection of *Pseudoalteromonas* confirms that phylogenetic analysis results in a number of clusters encompassing predominantly pigmented species or non-pigmented species (Ivanova et al. 2004). Strain S3431 was the single pigmented strain in the so-called non-pigmented clusters. Novel diversity might be represented in cluster III which consisted of strains with 98% or less 16S rRNA gene sequence similarity to *Pseudoalteromonas* type strains and formed a separate cluster in the phylogenetic analysis. However, these strains showed no antibacterial activity and no small molecule metabolites were detected. Such novel diversity could still represent untapped biotechnological potential, producing, e.g., enzymes or peptides with biological activity, as known for other non-pigmented *Pseudoalteromonas* (Hoyoux et al. 2001; Violot et al. 2005).

Chemical profiling of the strains detected an array of secondary metabolites. In addition to complementing our analysis of 16S rRNA gene sequences, it also demonstrated that some compounds (e.g., violacein, prodigiosin) were characteristic of a species and other compounds were produced by several species, and we also detected intra-species clusters of different secondary metabolite profiles. In a broad sense, the clustering based on 16S rRNA gene similarity agreed with the groups resulting from the chemophylogenetic analysis. However, some compounds were produced by organisms of different species that then clustered together using the secondary metabolites as basis. The chemical analysis separated the four isolated *P. luteoviolacea* strains into two distinct sub-groups, showing intra-species chemical diversity. The *P. luteoviolacea* strains produced violacein and

pentabromopseudilin which are active against gram-positive and gram-negative bacteria and the anti-staphylococcal agent indolmycin (Hornemann et al. 1971; Hurdle et al. 2004). Violacein and pentabromopseudilin have previously been detected in *P. luteoviolacea* (Gauthier 1976a; Laatsch and Pudleiner 1989), but to our knowledge, this is the first report of *Pseudoalteromonas* strains producing indolmycin (Månsson et al. 2010).

Within some species, all strains were consistently antibacterial. However, in others, such activity did not appear to be a consistent trait of the species. For instance, strains of the 16S cluster VI (*P. phenolica*) were heterogeneous in their ability to inhibit *Vibrio* in our assays, while all but one strain in the homogeneous cluster VII had identical metabolite profiles and all were inhibitory. Even more obvious was the heterogeneous chemical profiles within the *P. rubra* strains. All except one strain shared a chemical marker prodigiosin and/or RT 15.99 min but had major variations in 14 other compounds. This may in part be due to loss of ability to produce a compound. For instance, strain S2471 over time lost ability to produce the brominated indole (RT 13.78 min). Also, we note that the type strain DSM 6842 (ATCC 29570) did not in our culture produce prodigiosin which has been observed previously (Gauthier 1976b; Gauthier and Flatau 1976). The consistent bromoalterochromide production in the two species *P. piscicida* and *P. flavipulchra/maricaloris* (cluster VII) was expected (Speitling et al. 2007) and supported the high DNA sequence similarity between the two. This emphasizes the need to isolate and screen multiple strains from each species when bioprospecting within the genus *Pseudoalteromonas*, as even the homogeneous cluster VII contains one strain with a metabolite profile that does not share a single compound with the other strains in this cluster.

Several of the 26 detected peaks were known substances, with a majority known as antibacterials. These included violacein (Lichstein and Vandesand 1945), two bromopseudilins (Lovell 1966), two indolmycins (Werner 1980), four quinolines (Wratten et al. 1977), and prodigiosin (Kalesperis et al. 1975). Due to its very low aqueous solubility, violacein probably protects against predation rather than acts as a true antibiotic, and it has been shown to induce cell death in grazing organisms (Matz et al. 2008). Such compounds would be very beneficial for protection of a biofilm, which is likely how surface-associated *Pseudoalteromonas* would grow. The 14 compounds that could not be identified were mainly not identified due to poor ionization in ESI<sup>+</sup> and ESI<sup>-</sup> and/or several plausible candidates in Antibase2009. However, for chemotaxonomic studies, identity of the compounds is not necessary as long as they can be unambiguously identified between samples (Frisvad et al. 2008).

Within cluster VIII (*P. ruthenica*) and cluster II (*aurantialia/citrea*), we found examples where strains with highly similar

16S rRNA gene sequences (>99%) and with identical chemotaxonomy originated from geographically distinct locations. This latter observation is in agreement with studies on *Salinispora* biogeography and secondary metabolite production in which the authors show how strains of the marine bacterium *S. arenicola* isolated from worldwide locations are highly related and produce identical patterns of secondary metabolites (Jensen and Mafnas 2006; Jensen et al. 2007). In contrast, the *P. luteoviolacea* and *P. rubra* strains showed both local and global variations in their secondary metabolite profile, which one might speculate is due to adaptation to local specific niches.

In conclusion, we believe sampling from specific niches, e.g., biofilms on surfaces, to be of importance in discovery of novel secondary metabolites from the genus *Pseudoalteromonas*. While differences in metabolite patterns among species encourage isolation and screening of novel diversity, bioprospecting known *Pseudoalteromonas* species should not be ruled out. Investigation of multiple strains of one *Pseudoalteromonas* species can yield novel compounds due to intra-species variations within secondary metabolite profiles.

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## PAPER 2

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Vynne N, Månsson M, Nielsen KF, Gram L (2012)

Antibiotic chemoprofile and RecA and GyrB sequence analyses reveal three phlotypes within the marine *Pseudoalteromonas luteoviolacea*.

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1 Antibiotic chemoprofile and RecA and GyrB sequence analyses reveal three  
2 phlotypes within the marine *Pseudoalteromonas luteoviolacea*

3

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13

14

15 Running title: Chemoprofiles and phlotypes in *P. luteoviolacea*

16

1 **Abstract**

2 The bacterial species *Pseudoalteromonas luteoviolacea* produces antibiotic compounds and  
3 *Pseudoalteromonads* are, in general, known for their bioactivity. This has made them an interesting topic of  
4 research in the area of drug discovery. A major time sink in natural products discovery is the effort spent  
5 rediscovering known compounds, and methods to avoid this are in high demand. We recently found that  
6 four strains of *P. luteoviolaceae* indistinguishable by 16S rRNA gene sequence produced two different  
7 antibiotic profiles. The purpose of the present study was to determine if this sub-division was true for a  
8 larger group of *P. luteoviolaceae* isolates and to determine if it could be related to their phylogeny. These  
9 analyses would shed light on inter-species evolution and serve to determine if simple phylogenetic tools  
10 could assist in further discovery of bioactives in species already known to produce bioactive secondary  
11 metabolites. The 13 *P. luteoviolacea* strains divided into three chemoprofiles based on antibiotic  
12 production (violacein, violacein + pentabromopseudilin, violacein + indolmycin). Detailed phylogenetic  
13 analysis of GyrB and RecA translated amino acid sequences divided the strains into phylotypes related to  
14 antibacterial activity and production of the antibiotic compounds. Hence, we believe phylogeny within a  
15 bioactive species has the potential to be a useful tool in biodiscovery efforts. Additionally, the correlation  
16 between evolutionary groups and antibiotic production provides evidence that the antibiotic biosynthetic  
17 pathways are maintained under selective pressure and hence likely confer important benefits for the  
18 producer organism.

19

20 237 words.

## 1 Introduction

2

3 Many antibiotics used in treatment of infectious disease are of natural product origin, and despite high  
4 hopes for new drug discovery strategies, alternative approaches to drug discovery such as combinatorial  
5 chemistry has failed to adequately supply the drug pipeline (48). Therefore, we must revert to discovery of  
6 novel natural products capable of inhibiting or killing pathogenic bacteria (6). Screening of microorganisms  
7 (47) from extreme or underexplored environments(1) could be a promising approach as novel genetic  
8 diversity ensuring adaptation to extreme conditions may encode production of novel compounds. The  
9 marine environment is still considered an underexplored source of novel antimicrobial compounds and  
10 especially marine microorganisms are viewed as a potential source of novel antibiotic compounds  
11 (15,23,40). Indeed, marine bacteria belonging to the *Cyanobacteria* (60), *Actinobacteria* (49),  
12 *Pseudoalteromonas* (2,62), and the *Roseobacter* clade (4,28) produce compounds with interesting  
13 pharmacological properties.

14

15 Members of the genus *Pseudoalteromonas* are ubiquitous in the marine environment (65) and form two  
16 phylogenetic groups supported by analysis of 16S rRNA gene sequences (46,62). One group predominantly  
17 consists of non-pigmented pelagic bacteria with low or no antibacterial activity while the majority of  
18 species in the other group is pigmented and antagonistic bacteria typically isolated from marine biotic  
19 surfaces. The widespread occurrence of these bacteria is an indication of successful survival and  
20 competition strategies in which a trait such as production of antagonistic compounds could play an  
21 important ecological role (26). Consequently, the *Pseudoalteromonas* represent a promising target for  
22 biodiscovery efforts.

23

24 One of the main challenges in natural products discovery is the effort squandered rediscovering known  
25 compounds (9), hence so called 'dereplication' strategies to reduce the degree of rediscovery prior to

1 purification and structure elucidation steps are of outmost importance (29,50,55). One such strategy is  
2 early stage dereplication informed by microbial taxonomy (36,37,58). However, this is complicated by the  
3 inherent challenges of taxonomical classification (16) and more so, by the observation that closely related  
4 strains of the same species may produce different antibiotics (62,63) while strains of different genera may  
5 produce the same antibiotics as observed for e.g. the bioactive pigment violacein(18,39,64). Such cross-  
6 species production of compounds is likely due to lateral gene transfer of gene clusters responsible for  
7 biosynthesis of these metabolites (14,51). Nonetheless, screening of a taxonomically dereplicated  
8 collection of *Actinobacteria* led to discovery of a high number of novel compounds as compared to the  
9 strain throughput (20). Hence, it may possible to apply knowledge of bacterial systematics and taxonomy as  
10 a guide for efficient bioprospecting within other groups of bacteria. We previously reported (41,62) that  
11 five *Pseudoalteromonas luteoviolacea* strains produced two combinations of the three antibacterial  
12 compounds violacein (39), indolmycin (42), and pentabromopseudilin (PBP) (5) and accordingly may serve  
13 as a model to explore this hypothesis.

14

15 The aim of the present study was investigate phylogeny as a tool for dereplication in biodiscovery efforts  
16 and provide an evolutionary perspective on bacterial antagonism within the species *Pseudoalteromonas*  
17 *luteoviolacea*. This was achieved through detailed analysis of GyrB and RecA sequences coupled with  
18 chemical detection of three known antibacterial compounds; violacein, indolmycin, and  
19 pentabromopseudilin. We found that the chemoprofile was related to phylogenetic affiliation which  
20 validates phylogeny as an approach for dereplication of bacterial strains in a natural products perspective  
21 and offers evidence that the underlying biosynthetic pathways are maintained through the course of  
22 evolution.

23

## 24 **Materials and methods**

25

1 **Bacterial strains and culture conditions.** Thirteen *P. luteoviolacea* strains were used in this study (table 1).  
2 They originated from distinct geographical areas and were primarily isolated from surface water or algae.  
3 The strains were cultured in a marine minimal medium (MMM) (52) with 4.0 g/l mannose and 3.0 g/l  
4 casamino acids. All strains were incubated at 25 °C and 200 rpm agitation.

5  
6 **Assays for inhibition of bacterial growth.** *Vibrio anguillarum* 90-11-287 (57) and *Staphylococcus aureus*  
7 8325 were cultured in tryptic soy broth (Difco, USA). Well diffusion agar assays were carried out as  
8 previously described (28). The assay substrate contained 30 g/l Sea Salts (Sigma, USA) and 10 g/l agar. To  
9 support growth of *V. anguillarum* 4 g/l glucose and 3 g/l casamino acids was added. An additional 5 g/l  
10 peptone was added to *S. aureus* agar. The inter-species inhibition was tested by embedding each of the 13  
11 strains in agar and testing inhibition by sterile filtered culture of the same strains. The agar-substrate was  
12 modified for the inhibition assays targeting *Pseudoalteromonas luteoviolacea*: Glucose was substituted with  
13 4 g/l mannose, and due to temperature sensitivity of the *P. luteovioacea*, standard bacteriological agar was  
14 replaced with low gelling point agarose (Sigma, USA). The growth substrate for the assays was cooled to 37  
15 °C in a water bath before overnight bacterial culture was added, and the assays were subsequently  
16 performed as for *V. anguillarum* and *S. aureus*.

17  
18 **Analytical detection of antibacterial compounds.** Samples for LC-MS analyses were prepared from cultures  
19 in MMM extracted with ethyl acetate (EtOAc). Extracts were dried under nitrogen and redissolved in  
20 methanol (MeOH). LC-MS samples were analysed using an Agilent 1100 HPLC system with a diode array  
21 detector (Waldbronn, Germany) coupled to an LCT TOF mass spectrometer (Micromass, Manchester, UK)  
22 using a Z-spray electrospray (ESI) source. A Phenomenex Luna II C<sub>18</sub> column (50 mm ×2 mm, 3 µm) was used  
23 for separation, applying a linear acetonitrile (MeCN)-water (20 mM formic acid) 0.3 ml min<sup>-1</sup> gradient (15-  
24 100%) over 20 min at 40°C. For all LC-MS analyses, violacein and indolmycin were detected in positive  
25 ionisation mode (ESI<sup>+</sup>), while pentabromopseudilin was detected in negative mode (ESI<sup>-</sup>).

1  
2 **PCR amplification and sequencing.** DNA was purified from overnight *P. luteoviolacea* cultures using the  
3 NucleoSpin Tissue kit (Machery-Nagel, Germany) or QIAGEN Genomic-Tip G/100 (QIAGEN, USA) following  
4 the manufacturer's protocol. 16S rRNA genes from strains H33, H33S, NCIMB 1942, NCIMB 1944 and  
5 NCIMB 2035 were amplified by PCR, and *gyrB* and *recA* genes were amplified from all strains. One reaction  
6 consisted of 2.5 µl 10X hot start PCR buffer (Fermentas, Canada), 2.5 µl 2mM dNTP mix, 4 µl 25 mM MgCl<sub>2</sub>,  
7 0.8 µl 12.5 µM forward primer, 0.8 µl 12.5 µM reverse primer, 12.28 µl MilliQ H<sub>2</sub>O, 0.2 µl Maxima Hot Start  
8 *Taq* DNA polymerase (Fermentas, Canada) and 1 µl DNA template at 50 ng/µl for a total volume of 25 µl.  
9 The reactions were performed on an Applied Biosystems Veriti 96 well cycler. 16S rRNA genes were  
10 amplified according to [Porsby]. The primers and reaction conditions used for *gyrB* amplification were as  
11 described in (66). Primers and conditions for amplification of *recA* fragments were according to (53).  
12 Sequencing was done by Eurofins MWG Operon, Germany.

13  
14 **Phylogenetic analysis.** The 16S rRNA gene sequences were obtained from GenBank or by sequencing and  
15 aligned in MEGA5 using MUSCLE (59). The evolutionary history was inferred using the maximum likelihood  
16 method based on the Hasegawa-Kishino-Yano model (25), determined by MEGA5 as the best fitting model.  
17 The phylogenetic tree was constructed using MEGA5 default settings. For analysis of *recA* and *gyrB*  
18 sequences, an alignment was created for each gene in MEGA5 using MUSCLE. The alignments were curated  
19 manually and trimmed to be of equal length and in-frame for translation to amino acid sequence. The two  
20 alignments were concatenated and phylogenetic analyses were performed in MEGA5. Evolutionary  
21 relationships based on the encoded amino acid sequence were inferred using the maximum likelihood  
22 method with Dayhoff matrix-based distance calculations (56). A phylogenetic tree was generated and  
23 tested with 1000 bootstrap replications.

24

1 **Minimum inhibitory concentrations (MIC) of violacein and indolmycin.** MICs were determined for  
2 violacein (Sigma, USA) and indolmycin (Bioaustralis, Australia) as these compounds were commercially  
3 available. MIC assays were carried out in 96-well microtiter plates according to the guidelines by the clinical  
4 and laboratory standards institute (7), with minor modifications. *P. luteoviolacea* strains, *V. anguillarum* 90-  
5 11-287, and *S. aureus* 8325 were tested for sensitivity to indolmycin by determining the MIC. *P.*  
6 *luteoviolacea* was cultured in MMM with 4 g/l mannose and 3 g/l casamino acids, *V. anguillarum* in MMM  
7 with 4 g/l glucose and 3 g/l casamino acids, and *S. aureus* in MHB. Overnight bacterial cultures were diluted  
8 to 10<sup>5</sup> CFU/ml and 90 µl added per well. 10 µl of antibiotic solution was added to each well. Indolmycin was  
9 tested in serial two-fold dilution at final concentrations of 0.5 µg/ml to 128 µg/ml. Controls were included  
10 for no antibiotic and for ethanol solvent. The well containing the lowest concentration of antibiotic that  
11 had no visual bacterial growth after 48 hours corresponded to the MIC.

12

13 **Genbank nucleotide accession numbers.** 16S rRNA nucleotide sequences JQ250820-JQ250824, *gyrB*  
14 nucleotide sequences JQ280430 – JQ280442 and *recA* nucleotide sequences JQ280417-JQ280429.

15

## 16 **Results**

17

18 ***P. luteoviolacea* production of antibacterial compounds and antibacterial activity.** A collection of *P.*  
19 *luteoviolacea* strains were tested for the ability to inhibit bacterial growth using either live cells or sterile  
20 filtered culture supernatant. The strains were also tested for their ability to produce three known  
21 antibacterial compounds previously identified within the species (table 2). All 13 strains produced the  
22 purple antibacterial pigment violacein. Four strains did not produce other known antibiotics, three strains  
23 produced indolmycin and five strains produced PBP (table 2). No strain produced both indolmycin and PBP.  
24 Despite the violacein production by all strains, just 11 of the 13 strains inhibited *V. anguillarum* and *S.*  
25 *aureus* in the live cell assay. In the test of sterile filtered culture supernatants, only the three indolmycin

1 producing strains inhibited both *V. anguillarum* and *S. aureus*. Supernatants from nine strains inhibited *S.*  
2 *aureus*, and these strains produced indolmycin or PBP. The supernatant from the remaining four strains  
3 were not inhibitory against any of the target organisms and these were the four strains that produced  
4 neither indolmycin nor PBP. Sterile filtered culture supernatants of the *P. luteoviolacea* strains were also  
5 tested for intra-species inhibition (table S1). Five sterile filtered supernatants inhibited the full spectrum of  
6 *P. luteoviolacea* strains, including the producer strain itself: Two of the indolmycin producing strains  
7 (S4047-1 and S4054) and three of the PBP producers (S2607, S4060-1 and 2ta16). Four supernatants  
8 inhibited three to five *P. luteoviolacea* strains. Among these, interestingly the supernatants of strains H33  
9 and H33S, both unable to inhibit *V. anguillarum* or *S. aureus*, did inhibit three (H33) and five (H33S) strains  
10 respectively. Overall, however, the intra-species inhibition patterns did not correlate with the antibiotic  
11 production profile of the strains.

12  
13 **Phylogenetic analyses and assignment of phylotypes.** A 16S rRNA gene sequence and a GyrB-RecA based  
14 phylogenetic tree were created. The 16S rRNA sequences were similar at a level of >99% and the  
15 phylogenetic reconstruction showed no correlation to production of antibiotics (figure 1). In contrast, three  
16 phylotypes were identified in the GyrB-RecA phylogeny (figure 2; I, II, and III) and corroborated the division  
17 of *P. luteoviolacea* strains into groups with specific antibiotic production and bacterial inhibition. Phylotype  
18 I was synonymous with the six PBP producing strains. Within phylotype II, the indolmycin producer S4054  
19 placed itself as a representative of an ancestral state to the two other indolmycin producers and, on a  
20 separate branch, the strains H33 and H33S. Phylotype III was synonymous with the two strains NCIMB 1944  
21 and NCIMB 2035 which were not antagonistic.

22  
23 **MIC of known antagonistic compounds.** To investigate the potential role of indolmycin and violacein as  
24 antibacterial compounds, the MICs against *V. anguillarum*, *S. aureus*, and the 13 *P. luteoviolacea* strains  
25 were determined. Under these experimental conditions violacein did not inhibit any of the tested strains at

1 the concentrations included in the experiment; hence the MIC of violacein for all tested strains was > 128  
2 µg/ml. The MIC of indolmycin to *V. anguillarum* was 2 µg/ml. *S. aureus* was inhibited by indolmycin at all  
3 tested concentrations, resulting in a MIC of ≤ 0.5 µg/ml. None of the *P. luteoviolaceae*, irrespective of  
4 antibiotic profile, were inhibited by indolmycin and MIC was > 128 µg/ml

5

## 6 **Discussion**

7 *Pseudoalteromonas luteoviolacea* is a well-known producer of several bioactive secondary metabolites  
8 which are believed to play important ecological roles for the producer organism, such as protection against  
9 predation or bacterial antagonism. However, little is known of the phylogenetic distribution of antibiotic  
10 compounds within this species. We recently (Mansson et al. 2010, Vynne et al 2011) found that within a  
11 collection of just five strains two distinct antibiotic profiles were detected, prompting us to speculate if this  
12 reflected phylogenetic diversity or was a mere phenotypic variation. In the present study, we expand our  
13 investigation to a collection of 13 strains demonstrating three different antibiotic profiles within this  
14 species. We have attempted to further broaden our collection; however, were not able to obtain more  
15 strains as *P. luteoviolacea* under certain conditions is autoinhibitory (18) and several laboratories no longer  
16 had stock cultures. Our data demonstrate that biodiscovery processes can benefit from exploring several  
17 strains of the same species.

18

19 All strains produced violacein, a hallmark compound of the species; however, the data presented here and  
20 currently in preparation (10) indicates that the antibacterial activity of violacein is limited. Alternatively,  
21 violacein likely acts as a cell-associated anti-predation compound (44) which is more in line with its low  
22 solubility in water and its clear cell-association. Violacein is not present in a simple sterile filtered  
23 supernatant and these were only antibacterial if harvested from strains producing either indolmycin or PBP.  
24 The indolmycin or PBP containing supernatants were equally inhibitory towards *S. aureus*, whereas only  
25 indolmycin containing supernatants inhibited the Gram-negative *V. anguillarum*. This is in agreement with

1 previous studies describing pentabromospeudilin as a compound targeting Gram-positive bacteria (5),  
2 whereas indolmycin, previously only isolated from *Streptomyces* species (35,61), inhibits both Gram-  
3 positive and Gram-negative bacteria (35) and is very potent against staphylococci (31). The genetic basis of  
4 the biosynthetic pathways of PBP and indolmycin is not known, but the available mechanistic insight  
5 (24,30,54) suggests that it is two distinct biosynthetic pathways with no common precursor. However, as  
6 indolmycin and violacein both are tryptophan derived it is not unlikely that their biosynthetic pathways are  
7 interlinked at an early stage. Interestingly, the strains H33 and H33S did not produce indolmycin or PBP, yet  
8 were active in the live cell assay and in intra-species inhibition. This could potentially be explained by some  
9 degree of antibacterial activity of violacein or violacein analogues present in the growing colony, or it could  
10 be related to macromolecular antibacterial substances as described in strains CPMOR-1 and CPMOR-2.  
11 CPMOR-1 and CPMOR-2 produce a macromolecular L-amino acid oxidase with antibiotic activity (19), but in  
12 contrast to H33 and H33S, CPMOR-1 and CPMOR-2 did not cause intra-species inhibition. Therefore it is  
13 also possible that other as yet undiscovered antibiotics are produced by H33 and H33S.

14  
15 Our analysis of 16S rRNA gene sequences (figure 1) resolved two clades with no apparent relation to  
16 antibiotic production. However, the resolving power of the 16S rRNA gene is limited when performing  
17 phylogenetic analyses below the species level and for many species, studies have shown the usefulness of  
18 phylogenetic analyses of housekeeping genes such as *recA* and *gyrB*. Hence, analysis of antibacterial  
19 secondary metabolite production and phylogenetic reconstruction based on the housekeeping genes *recA*  
20 and *gyrB* divided the 13 strains into three phylotypes with specific production of antibiotics (figure 2). Such  
21 a correlation among taxonomic units and secondary metabolite synthesis is well known from the world of  
22 fungal natural products (17) and has also been reported for a marine actinobacteria (34). Analysis of *GyrB*  
23 and *RecA* sequences provides a clearer resolution of taxonomy than 16S rRNA gene sequences when  
24 comparing strains at or below the species level(8,27,67), which may explain why phylotype specific

1 secondary metabolite production was only partially observed in our previous analysis of 16S rRNA gene  
2 based phylogeny and chemotypes in 101 *Pseudoalteromonas* strains (62).

3  
4 The phylogenetic analysis placed the indolmycin producer S4054 near the divergence point of the non-  
5 indolmycin producers H33 and H33S. This indicates that indolmycin production is an ancestral trait, and we  
6 speculate that H33 and H33S may have lost or silenced the indolmycin biosynthetic pathway to rely on a  
7 different antagonistic compound in microbial competition since inhibition was observed in the live cell  
8 assay, or that the divergence point was shortly before acquisition of the indolmycin producing trait. This  
9 raises the question of which taxonomical level to consider when searching for novel chemical diversity, as  
10 selection of biological material based on 16S rRNA gene sequences may exclude chemical diversity (33). It  
11 is tempting to speculate that phylotype III employs a different strategy when colonizing and competing in  
12 the marine environment as these strains showed no inhibition in live cell assays or tests of sterile  
13 supernatants. However, antagonism may simply be silenced under the tested growth conditions, as it is  
14 known that environmental factors such as host components (3) or microbial interactions (11,45) may  
15 facilitate the production of antagonistic compounds. Further insight could be gained via whole genome  
16 sequencing in order to search for potential clusters of genes involved in e.g. polyketide synthesis or non-  
17 ribosomal peptide synthesis (22).

18  
19 Often, genes involved in biosynthetic pathways occur grouped within the bacterial genome and are  
20 subjects of acquisition or reduction events (13,32,38). Production of antagonistic compounds that play a  
21 key ecological role for the producer organism is expected to be under strong selective pressure, as was  
22 observed for phylotype I and could be argued for in the indolmycin sub-population in phylotype II. This is  
23 consistent with the model of gene fixation in the complexity hypothesis (32) in which genes with a positive  
24 selection pressure are maintained at a high occurrence within a taxonomical unit as observed for three  
25 closely related *Salinispora* species (34). Species specific chemotypes are known in the marine environment

1 from e.g. the actinobacterial genus *Salinispora* (34) and were suggested to reflect adaptation to specific  
2 environmental niches. Altogether, this suggests that the compounds indolmycin and PBP play important  
3 roles in the ecology of the producer organism, as their production can be traced through a phylogenetic  
4 reconstruction of closely related strains and thus are maintained on an evolutionary scale. Although the  
5 present study has no data to support previous reports of the antibacterial activity of violacein, we believe  
6 that the compound does play a key role in *P. luteoviolacea* ecology as for instance protection against  
7 predatory microorganisms (43,44).

8  
9 Based on the limit number of strains in this study, no apparent correlation among antibiotic production and  
10 geographical location was observed within the *P. luteoviolacea* strains, although the majority of the  
11 antagonistic strains were isolated from surfaces and, specifically, all indolmycin-producing strains were  
12 isolated from macroalgae or seaweed. The apparent lack of correlation among antibiotic production and  
13 geographic origin is in contrast to observations within the marine actinobacterium *Salinispora arenicola*,  
14 where biogeographic patterns including local endemism were observed among polyketide synthases likely  
15 involved in synthesis of bioactive secondary metabolites (12). Two of the *P. luteoviolacea* strains (4054  
16 and 4060) had different antibiotic profile and were originally isolated from the same sample (21). No  
17 systematic intra-species inhibition pattern was observed and all *P. luteoviolacea* strains, irrespective of  
18 antibiotic production profile, were resistant to indolmycin. We speculate that the species gains an  
19 ecological advantage by colonizing with strains producing different antibiotics, hence broadening the  
20 competitive ability against other species.

21

22 In summary, we found that the marine bacterium *Pseudoalteromonas luteoviolacea* produces the  
23 bioactives violacein, indolmycin, and PBP. Violacein as a pure compound did not inhibit any of the tested  
24 bacteria, whereas indolmycin inhibited *V. anguillarum* and *S. aureus* but not the *P. luteoviolacea* strains.  
25 We found that the antibiotic profile was correlated to – and hence could have been predicted by – the

1 phylogenetic analyses. This offered a glimpse of the evolutionary stability of the underlying biosynthetic  
2 pathways and provides a method for dereplicating bacterial strains as part of a natural product screening  
3 effort.

4

5

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2 Reference List  
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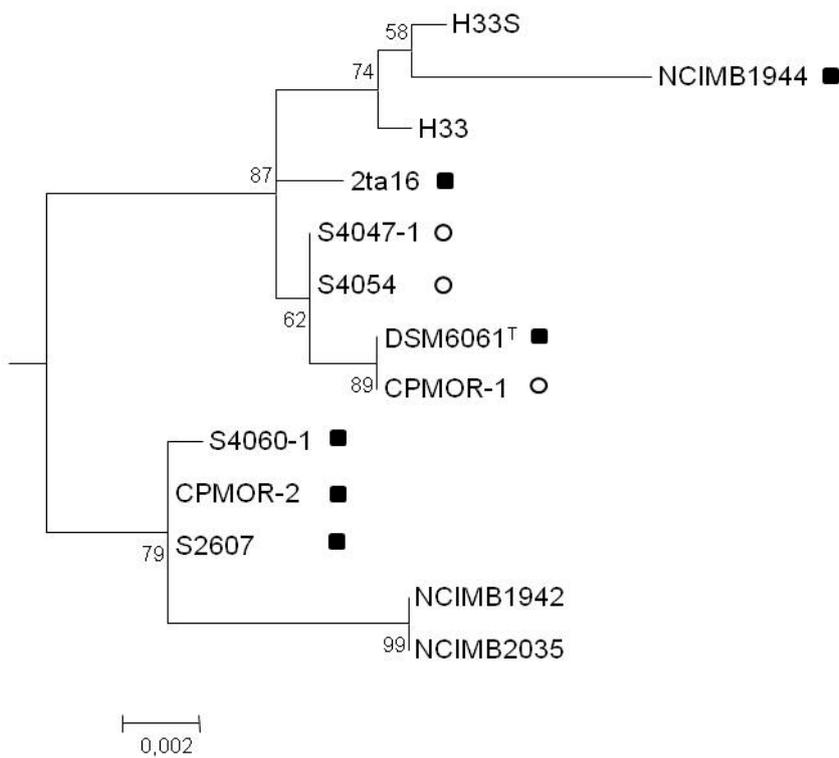
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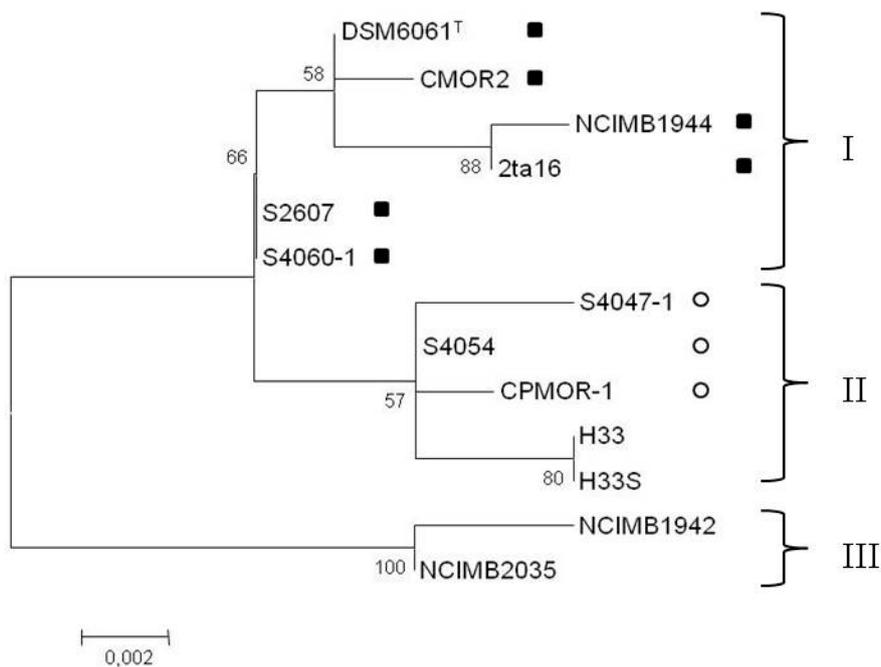
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**Figure 1:** Phylogenetic analysis of 16S rRNA gene sequences in MEGA5 (59) using *Pseudoalteromonas atlantica* (accession no. X82134) as outgroup (not shown in tree). The 16S rRNA gene sequences were aligned using MUSCLE, and the evolutionary history inferred using the maximum likelihood method based on the Hasegawa-Kishino-Yano model (25). A phylogenetic tree was constructed using the default MEGA5 settings. Scale bar: substitutions per site. Closed boxes: pentabromopseudilin producer, open circles: indolmycin producer. All strains produce violacein.



**Figure 2:** Phylogenetic analysis of concatenated RecA and GyrB amino acid sequences of 13 strains based on the maximum likelihood method. The evolutionary history was inferred by using the maximum likelihood method based on the Dayhoff matrix model(56). The tree with the highest log likelihood (-1597,5915) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 1000 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (59) using a discrete Gamma distribution to model evolutionary rate differences among sites (5 categories (+G, parameter = 0,1000)). Clades identified as phylotypes are indicated by roman numerals I-III. Scale bar: substitutions per site. Closed boxes: pentabromopseudilin producer, open circles: indolmycin producer. All strains produce violacein.

**Table 1:** *P. luteoviolacea* strains used in this study. The phylogenetic position of all strains within the *Pseudoalteromonas luteoviolacea* clade was verified by analysis of partial 16S rRNA gene sequences (>1200 bp, data not shown).

Strain name	Origin	Source
DSM 6061 (T)	Mediterranean, Nice	Surface water
S2607	Pacific, Eastern Australia	Rock surface
S4060-1	Pacific, Costa Rica	Seaweed
2ta16	Florida Keys, USA	<i>M. annularis</i> coral
CPMOR-2	Mediterranean, Murcia	Surface water
NCIMB1944	Mediterranean, Nice	Surface water
S4047-1	Pacific, Costa Rica	Seaweed
S4054	Pacific, Costa Rica	Seaweed
CPMOR-1	Mediterranean, Murcia	Macroalgae
H33	Sydney, Australia	Unknown
H33S	Sydney, Australia	Unknown
NCIMB1942	Mediterranean, Nice	Surface water
NCIMB2035	Mediterranean, Nice	Surface water

**Table 2:** Inhibitory activity of *P. luteoviolacea* strains in the live cell and supernatant well diffusion agar assays and production of three known antibacterial compounds in marine minimal medium cultures. The production of antibacterial compounds was determined by LC-MS analysis. *V. anguil.* = *Vibrio anguillarum*. **x**: Inhibition or compound production, respectively. **-**: No inhibition or compound production.

Strain	Live cell assay inhibition		Inhibition by sterile supernatant		Antibacterial compounds produced			Phylo type
	<i>V. anguil.</i>	<i>S. aureus</i>	<i>V. anguil.</i>	<i>S. aureus</i>	Violacein	Pentabromo-pseudilin	Indolmycin	
DSM6061 <sup>T</sup>	<b>x</b>	<b>x</b>	-	<b>x</b>	<b>x</b>	<b>x</b>	-	I
S2607	<b>x</b>	<b>x</b>	-	<b>x</b>	<b>x</b>	<b>x</b>	-	I
S4060-1	<b>x</b>	<b>x</b>	-	<b>x</b>	<b>x</b>	<b>x</b>	-	I
2ta16	<b>x</b>	<b>x</b>	-	<b>x</b>	<b>x</b>	<b>x</b>	-	I
CPMOR-2	<b>x</b>	<b>x</b>	-	<b>x</b>	<b>x</b>	<b>x</b>	-	I
NCIMB 1944	<b>x</b>	<b>x</b>	-	<b>x</b>	<b>x</b>	<b>x</b>	-	I
S4047-1	<b>x</b>	<b>x</b>	<b>x</b>	<b>x</b>	<b>x</b>	-	<b>x</b>	II
S4054 WT	<b>x</b>	<b>x</b>	<b>x</b>	<b>x</b>	<b>x</b>	-	<b>x</b>	II
CPMOR-1	<b>x</b>	<b>x</b>	<b>x</b>	<b>x</b>	<b>x</b>	-	<b>x</b>	II
H33	<b>x</b>	<b>x</b>	-	-	<b>x</b>	-	-	II
H33s	<b>x</b>	<b>x</b>	-	-	<b>x</b>	-	-	II
NCIMB 1942	-	-	-	-	<b>x</b>	-	-	III
NCIMB 2035	-	-	-	-	<b>x</b>	-	-	III

301

302

- 1 **Table S1:** Intra-species inhibition by *P. luteoviolacea* sterile supernatant in well diffusion agar assays. Substrate blanks were included and did not
- 2 inhibit any organism. +: inhibition, (+): weak inhibition, -: no inhibition.

Producer strain	Inhibition (+) of target strain												
	DSM6061 (T)	S2607	S4047-1	S4054	S4060-1	2ta16	CPMOR-1	CPMOR-2	NCIMB 1942	NCIMB 1944	NCIMB 2035	H33	H33S
DSM6061 <sup>T</sup>	-	+	-	-	+	-	-	-	-	-	+	-	-
S2607	+	+	+	+	+	+	+	+	+	+	+	+	+
S4060-1	+	+	+	+	+	+	+	+	+	+	+	+	+
2ta16	+	+	+	+	+	+	+	+	+	+	+	+	+
CPMOR-2	-	-	-	-	-	-	-	-	-	-	-	-	-
NCIMB 1944	(+)	-	(+)	(+)	-	-	-	-	-	-	-	-	-
S4047-1	+	+	+	+	+	+	+	+	+	+	+	+	+
S4054	+	+	+	+	+	+	+	+	+	+	+	+	+
CPMOR-1	-	-	-	-	-	-	-	-	-	-	-	-	-
H33	-	-	-	-	+	-	-	-	-	-	+	+	-
H33S	-	+	-	-	+	+	-	-	-	-	+	+	-
NCIMB 1942	-	-	-	-	-	-	-	-	-	-	-	-	-
NCIMB 2035	-	-	-	-	-	-	-	-	-	-	-	-	-

## PAPER 3

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Vynne N, Christiansen G, Gram L (2012)

*Pseudoalteromonas galathea* S3431 sp. nov., a novel *Pseudoalteromonas* species isolated from a deep-sea polychaete

Manuscript in preparation.

1 ***Pseudoalteromonas galathea* S3431 sp. nov. , a novel *Pseudoalteromonas* species**  
2 **isolated from a deep-sea polychaete**

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15 December 26<sup>th</sup> 2011

16

1 **Abstract**

2 A novel aerobic marine bacterium was isolated from swab samples of an unidentified polychaete  
3 near Canal Concepción, Chile. The strain was gram negative with a polar flagellum, and it grew  
4 from 5 to 30 °C. Growth required a NaCl concentration of 1 to 6% (w/v) and pH 5.5-8.9, and a wide  
5 range of carbohydrates were utilized as growth substrates. The main components of the total  
6 cellular fatty acids were summed feature 3 (C<sub>16:1</sub> ω7c/C<sub>16:1</sub> ω6c, 35.82%), C<sub>16:0</sub> (20.43%) and C<sub>17:1</sub>  
7 ω8c (10.85%). The DNA G+C content of the strain was 39.6 mol%. The most similar strain was  
8 *P. arctica* LMG 23753<sup>T</sup> (99.7%), and 16S rRNA gene sequence identities ranged from 92.6% to  
9 99.7% when compared to *Pseudoalteromonas* type strains. DNA-DNA reassociation values showed  
10 less than 70% genomic DNA relatedness to *Pseudoalteromonas* type strains. Based on the  
11 available phylogenetic and phenotypic data, this isolate represents a novel species of the genus  
12 *Pseudoalteromonas*. The name *Pseudoalteromonas galathea* is proposed and the type strain is  
13 S3431<sup>T</sup> = LMGXXXXX = DSM XXXX (to be added when/if accepted).

14

15

1 The genus *Pseudoalteromonas* contains several species that produce different bioactive  
2 compounds, and it has been found that for most species pigmentation co-occurs with production  
3 of bioactive metabolites (Bowman, 2007; Egan *et al.*, 2002; Vynne *et al.*, 2011). Among the known  
4 bioactives produced by pigmented members of the genus are antifouling, anti-bacterial and  
5 cytotoxic compounds, however, also non-pigmented species may be of biotechnological interest  
6 due to the production of novel enzyme activities (Bowman, 2007).

7 During a research cruise (Galathea 3), bacteria were collected from marine environments and  
8 screened for antibacterial activity (Gram *et al.*, 2010). Of the isolated strains, more than one  
9 hundred were identified by 16S rRNA gene sequence similarity as *Pseudoalteromonas* (Gram *et al.*,  
10 2010; Vynne *et al.*, 2011). On February 3<sup>rd</sup> 2007, a swab sample was obtained from an unidentified  
11 polychaete near Canal Concepción, Chile (-50.4498 °N, -74.8912 °E). From subcultures of this  
12 sample, strain S3431 was isolated due to production of an intense black pigment. Later analysis  
13 would show a high level of 16S rRNA sequence similarity to strains of the so called non-pigmented  
14 group of the genus *Pseudoalteromonas* (Gauthier *et al.*, 1995), in clear contrast to the intense  
15 black pigment produced by this strain. The most related type strain was *P. arctica* LMG 23753<sup>T</sup>  
16 (99.7% identical) as determined by a BLAST comparison of 16S rRNA gene sequence of S3431 and  
17 *Pseudoalteromonas* type strains. Several other type strains also had >99% identity and were  
18 included in this study. Further taxonomic and physiological analysis of this new strain determined  
19 that it represents a novel species within the *Pseudoalteromonas* genus.

20 Strain S3431 was routinely cultured for morphological and physiological characterisation in marine  
21 broth 2216 (Difco, USA) and on marine agar 2216 (Difco, USA) at 25°C. A marine minimal medium  
22 (MMM) containing no carbon source was used as substrate for BioLog assays (Östling *et al.*, 1991).  
23 Temperature requirements for growth were tested in MB 2216 from 5°C to 30°C in 5°C intervals,  
24 and at 37°C. NaCl requirements were tested on ½YT agar medium containing 2 g/l yeast extract,  
25 1.25 g/l tryptone, 10 g/l agar and NaCl in concentrations of 0% – 9% (w/v). The pH range  
26 supporting growth was determined in MB, pH 4.2 – 10.3.

27

28 Cell shape and motility was investigated in wet mounts using an Olympus BX51 phase contrast  
29 microscope, while the presence of flagella was determined by transmission electron microscopy

1 (TEM). For TEM, colony mass from colonies grown on MA was resuspended in phosphate buffered  
2 saline, pH 7.4. Seven  $\mu\text{l}$  of this suspension were placed on a 300-mesh Ni-grid coated with carbon  
3 film glow discharged for 30 seconds. The grid was washed using 2 drops of double distilled  $\text{H}_2\text{O}$   
4 and stained with 3 drops of PTA pH 6.9 for 15 seconds, after which the grid was dried by filter  
5 paper. TEM was performed at 60 keV on a JEOL1010 transmission electron microscope fitted with  
6 a digital camera.

7 Gram-testing was done using the 3% KOH method and aminopeptidase strips (BactiDent). Catalase  
8 activity was tested using the 3%  $\text{H}_2\text{O}_2$  method, and oxidase activity was tested using BD BBL  
9 DrySlide. The ability of the strain to ferment glucose was tested using Hugh & Leifsons media  
10 modified for marine microorganisms by adding 2.0% Instant Ocean salts (Aquarium Systems, Inc.,  
11 Sarrebourg, France) (Hugh & Leifson, 1953). BioLog GN2 microtiterplates were used to test for  
12 carbon assimilation: S3431 was grown on MA overnight and colony mass was resuspended to  
13  $\text{OD}_{600} = 1.0$  in MMM with no carbon source, supplemented with 5 mM thioglycolate. 150  $\mu\text{l}$  of this  
14 suspension was added to each well of the GN2 microplate. The plate was manually read after 10  
15 days of incubation at 25°C.

16 Hemolytic activity was tested on Blood Agar Base (Oxoid) with 5% defibrinated calf blood.  
17 Production of enzymes was tested by spotting colony mass of S3431 on medium consisting of  
18 28.75 ml/l buffer (85% phosphoric acid 0.08 M, boric acid 0.08 M, glacial acetic acid 0.08 M), 30 g/l  
19 Instant Ocean salts and 10 g/l agar and distilled water to 1000 ml. The pH of the medium was  
20 adjusted to 6.0 and enzyme substrate was added to a concentration of 0.1% (w/v). Azurine-  
21 crosslinked (AZCL)-amylose, AZCL-curdlan, AZCL-galactan, AZCL-rhamnogalacturon I, AZCL-xylose  
22 and AZCL-dextran were tested. Azo-avicel was used as substrate to test production of endo-  
23 cellulases. Enzyme activity was detected by the presence of a colored zone in the agar  
24 surrounding the colony. AZCL-substrates and azo-avicel were purchased from Megazyme, Ireland.  
25 Production of caseinase activity was tested on agar plates containing 100 g/l skim milk powder  
26 (Difco), 30 g/l Sea Salts (Sigma) and 15 g/l agar. Chitinase production was assayed on agar plates  
27 containing 0.5 g/l peptone, 0.1 g/l yeast extract, 0.01 g/l  $\text{FePo}_4$ , 30 g/l Sea Salts (Sigma) and 0.2%  
28 colloidal chitin (Weyland *et al.*, 1970).

1 The *Pseudoalteromonas* sp. S3431 16S-rRNA gene sequence was analysed using BLAST to  
2 determine closely related species. A phylogenetic tree was created based on *Pseudoalteromonas*  
3 type strain 16S-rRNA gene sequences. These were aligned using MUSCLE and a maximum  
4 likelihood tree was created using the Kimura 2-parameter model and 1000 bootstrap replications.  
5 Determination of GC-mol% of genomic DNA, cellular fatty acid profiling and DNA-DNA  
6 reassociation analyses were carried out by the DSMZ.

7

8 Bacterial cells of the novel isolate were Gram-negative, motile rods with one polar flagella as  
9 described for members of the genus *Pseudoalteromonas* (figure 1) (Mikhailov *et al.*, 2002). The  
10 cells were approximately 2.2 – 3.0 µm long and 0.7 – 0.8 µm wide. The isolate grew at 5 to 30°C,  
11 but not at 35°C. No sporulation was observed. Growth was observed at pH 5.5 but not pH 4.3, and  
12 at pH 8.9 but pH 9.7. The strain was capable of growth on ½YT agar containing 1 to 6% NaCl, but  
13 not in 0.5 or 7% NaCl. The strain was oxidase and catalase positive, and capable of oxidative but  
14 not fermentative glucose metabolism. Membrane vesicles appeared on several cells as evidenced  
15 by TEM, which was previously observed in other *Pseudoalteromonas* species (Nevot *et al.*, 2006).

16 Carbon source assimilation in the BioLog GN2 microplate system showed that, in addition to the  
17 carbon sources listed in table 1, S3431 was able to utilize α-cyclodextrin, dextrin, glycogen, tween  
18 40, tween 80, D-Cellobiose, gentiobiose, lactulose, D-raffinose, acetic acid, D-galacturonic acid, α-  
19 ketoglutaric acid, propionic acid, succinic acid, L-alaninamide, L-alanine, L-alanyl-glycine, L-  
20 asparagine, L-glutamic acid, glycy-L-glutamic acid, L-proline, L-serine, L-threonine, inosine, uridine,  
21 α-D-glucose-6-phosphate and D-glucose-6-phosphate as sole carbon sources.

22 The novel strain produced amylase but not curdlanase, galactanase, rhamnogalacturonase,  
23 xylanase or dextranase. No endo-cellulases were produced. Chitinase production was not  
24 detected, however agarolytic activity was observed on both chitin plates and agar medium  
25 containing 0.5 g/l peptone, 0.1 g/l yeast extract, 0.01 g/l FePO<sub>4</sub>·H<sub>2</sub>O and 30 g/l Sea Salts (Sigma).  
26 Caseinase activity was observed as clearing zones on skim milk agar.

27 The 16S-rRNA gene sequence was compared to 16S-rRNA sequences from *Pseudoalteromonas*  
28 type strains using the BLAST tool. The most similar strain was *P.arctica* LMG 23753<sup>T</sup> (99.7%), and

1 identities ranged from 92.6% to 99.7%. In a minimum evolution phylogenetic tree, the strain  
2 placed itself firmly within the so called non-pigmented group (supplementary figure 1), in contrast  
3 to the intense black pigment produced by this strain.

4 The DNA-DNA reassociation values of S3431 to type strains with 99% 16S rRNA identity are shown  
5 in table 2, and were below the 70% recommended limit for delineating new species (Wayne *et al.*,  
6 1987). G+C content in genomic DNA was 39.6 mol%, which is in range with other  
7 *Pseudoalteromonas* species (Gauthier *et al.*, 1995). The main cellular fatty acids were summed  
8 feature 3 (C<sub>16:1</sub> ω7c/C<sub>16:1</sub> ω6c, 35.82%), C<sub>16:0</sub> (20.43%) and C<sub>17:1</sub> ω8c (10.85%) as shown in table 3  
9 which confirms the position of strain S3431 within the genus *Pseudoalteromonas* (Ivanova *et al.*,  
10 2000).

11

12 Based on the available phenotypic and phylogenetic information, strain S3431 should be  
13 recognized as a new species within the genus *Pseudoalteromonas* and the name  
14 *Pseudoalteromonas galathea* sp. nov. is proposed.

15

#### 16 **Description of *Pseudoalteromonas galathea* sp. nov.**

17 *Pseudoalteromonas galathea* (ga.la.the'ae. N.L. n. galathea, referring to the research expedition on  
18 which the type strain was first isolated).

19 Cells are motile straight rods with polar flagella, 2.2 – 3.0 μm long and 0.7 – 0.8 μm wide. The cells  
20 are Gram-negative, non-spore forming, strictly aerobe and mainly occur as single cells. When  
21 grown on MA 2216 at 25 °C, the strain forms raised circular black to dark brown colonies, which  
22 appear smooth and shiny. A brown pigment diffusing into the agar is produced. Growth occurs at 4  
23 – 30 °C, with no growth at 37 °C. Growth is observed within a pH range of 5.5-8.9. Substrate NaCl  
24 content from 1-6% supports growth. The strain utilizes D-glucose, D-mannose, D-galactose,  
25 maltose, sucrose, melibiose, lactose, succinate, D-gluconate, D-mannitol, sorbitol, citrate, xylose,  
26 trehalose, acetate, L-arginine, α-cyclodextrin, dextrin, glycogen, tween 40, tween 80, D-cellobiose,  
27 gentiobiose, lactulose, D-raffinose, acetic acid, D-galacturonic acid, α-ketoglutaric acid, propionic

1 acid, succinic acid, L-alaninamide, L-alanine, L-alanyl-glycine, L-asparagine, L-glutamic acid, glycy-  
2 L-glutamic acid, L-proline, L-serine, L-threonine, inosine, uridine,  $\alpha$ -D-glucose-6-phosphate and D-  
3 glucose-6-phosphate as sole carbon sources. It does not grow on D-fructose, sorbitol, *N*-  
4 acetylglucosamine or pyruvate. The strain produces amylase and hydrolyzes agar and casein, but is  
5 not hemolytic. The primary cellular fatty acids are summed feature 3 ( $C_{16:1} \omega 7c/C_{16:1} \omega 6c$ , 35.82%),  
6  $C_{16:0}$  (20.43%) and  $C_{17:1} \omega 8c$  (10.85%).

7 The type strain is S3431<sup>T</sup> (= DSM XXXXX = LMG XXXXXX, to be added when accepted), isolated from  
8 a marine polychaete in Canal Concepción, Chile. The DNA G+C mol content of the type strain is  
9 39.6%.

10

## 11 **Acknowledgements**

12 We thank Dr. Bernhard Schink for ensuring a correct specific epithet. This study was supported by the  
13 Programme Commission on Health, Food and Welfare under the Danish Council for Strategic Research. The  
14 present work was carried out as part of the Galathea 3 expedition under the auspices of the Danish  
15 Expedition Foundation. This is Galathea 3 contribution no. pXXXXXX (to be added if/when accepted)

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- 13

- 1 **Figure 1:** TEM of cells of *Pseudoalteromonas galathea* S3431 showing the polar flagellum.
- 2 Additionally, vesicle-like structures appear near the cell membrane.



**Table 1:** Characteristics of *Pseudoalteromonas* type strains. 1: *P. galathea* S3431, 2: *P. arctica*<sup>T</sup>, 3: *P. translucida*<sup>T</sup>, 4: *P. haloplanktis*<sup>T</sup>, 5: *P. agarivorans*<sup>T</sup>, 6: *P. aliena*<sup>T</sup>, 7: *P. atlantica*<sup>T</sup>, 8: *P. carrageenovora*<sup>T</sup>, 9: *P. distincta*<sup>T</sup>, 10: *P. elyakovii*<sup>T</sup>, 11: *P. espejana*<sup>T</sup>, 12: *P. issachenkonii*<sup>T</sup>, 13: *P. nigrifaciens*<sup>T</sup>, 14: *P. paragorgicola*<sup>T</sup>, 15: *P. tetraodonis*<sup>T</sup>, 16: *P. undina*<sup>T</sup>.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
<b>DNA G-C content (mol%)</b>	39.6	39	46.3	43.3	43.8	41-43	41.2	39.5	43.8	38.5	43	43	39-41	41.1	41.5	42.2
<b>Growth at:</b>																
4 °C	+	+	+	-	-	+	-	(+)	-	v	-	+	+	+	+	+
30 °C	+	-	+	v	+	+	+	+	+	+	+	+	+	+	+	ND
40°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1% NaCl	+	+	+	+	+	v	+	+	+	+	+	+	+	+	+	+
3-6% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8% NaCl	-	+	-	+	+	-	-	+	+	+	+	+	+	-	+	+
9% NaCl	-	+	-	+	+	-	-	+	+	+	+	+	+	-	+	+
10% NaCl	-	-	-	+	-	-	-	+	+	+	+	+	+	-	+	+
<b>Enzymatic activity:</b>																
Catalase positive	+	ND	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase positive	+	ND	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Amylase	+	-	+	v	+	+	+	-	-	+	+	-	v	+	-	v
Caseinase	+	+	+	+	+	v	+	+	-	ND	ND	+	-	+	ND	ND
Agarase	+	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-
Chitinase	-	ND	-	v	ND	-	-	-	-	-	-	+	-	-	-	+
<b>Utilization of:</b>																
D-Glucose	+	+	-	+	+	v	+	+	+	+	+	+	+	ND	+	+
L-Arabinose	-	ND	ND	-	-	ND	-	-	-	-	-	ND	ND	ND	ND	v
D-Mannose	+	-	ND	+	-	-	+	-	+	+	v	-	v	ND	-	-
D-Galactose	+	+	+	v	-	-	+	v	-	+	+	+	+	+	+	-
D-Fructose	-	-	ND	+	-	-	+	+	+	+	v	+	+	ND	-	-
Maltose	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+
Sucrose	+	+	ND	v	-	ND	+	+	-	+	+	+	v	ND	+	+
Melibiose	+	+	-	-	-	-	+	+	+	v	+	+	+	-	-	-
Lactose	+	-	-	-	-	-	+	+	+	v	+	+	+	-	-	-
D-Gluconate	+	+	ND	-	-	-	-	-	-	-	-	-	+	ND	-	-
Glycerol	+	-	ND	-	-	-	+	+	+	-	v	-	+	ND	-	-
Succinate	+	-	ND	v	-	-	+	+	-	+	-	+	+	ND	v	+
D-Mannitol	+	+	+	v	+	ND	+	+	-	+	+	+	-	+	-	-
Sorbitol	-	ND	ND	-	ND	-	-	-	-	-	-	-	v	-	-	-
Citrate	+	-	-	+	-	-	v	+	+	v	+	+	+	-	+	-
N-Acetylglucosamine	-	-	ND	-	-	+	-	-	-	-	-	-	ND	ND	ND	+
Xylose	+	-	-	-	ND	-	-	-	-	+	+	-	v	ND	-	-
Trehalose	+	-	ND	-	ND	-	+	-	-	-	+	-	+	ND	-	v
Acetate	+	-	ND	+	-	-	+	+	+	v	+	+	+	ND	+	+
Pyruvate	-	+	ND	+	-	-	+	+	+	+	+	+	+	ND	+	+
L-Arginine	+	ND	-	-	ND	ND	-	-	ND	-	+	ND	v	-	-	v

**Table 2:** Per cent DNA-DNA similarity in 2X SSC at 66°C {Vynne, 2012 455 /id}. All related type strains have less than 70% DNA-DNA similarity to S3431, which is the accepted threshold for species delineation {Wayne, 1987 152 /id}.

WIP: Work in progress.

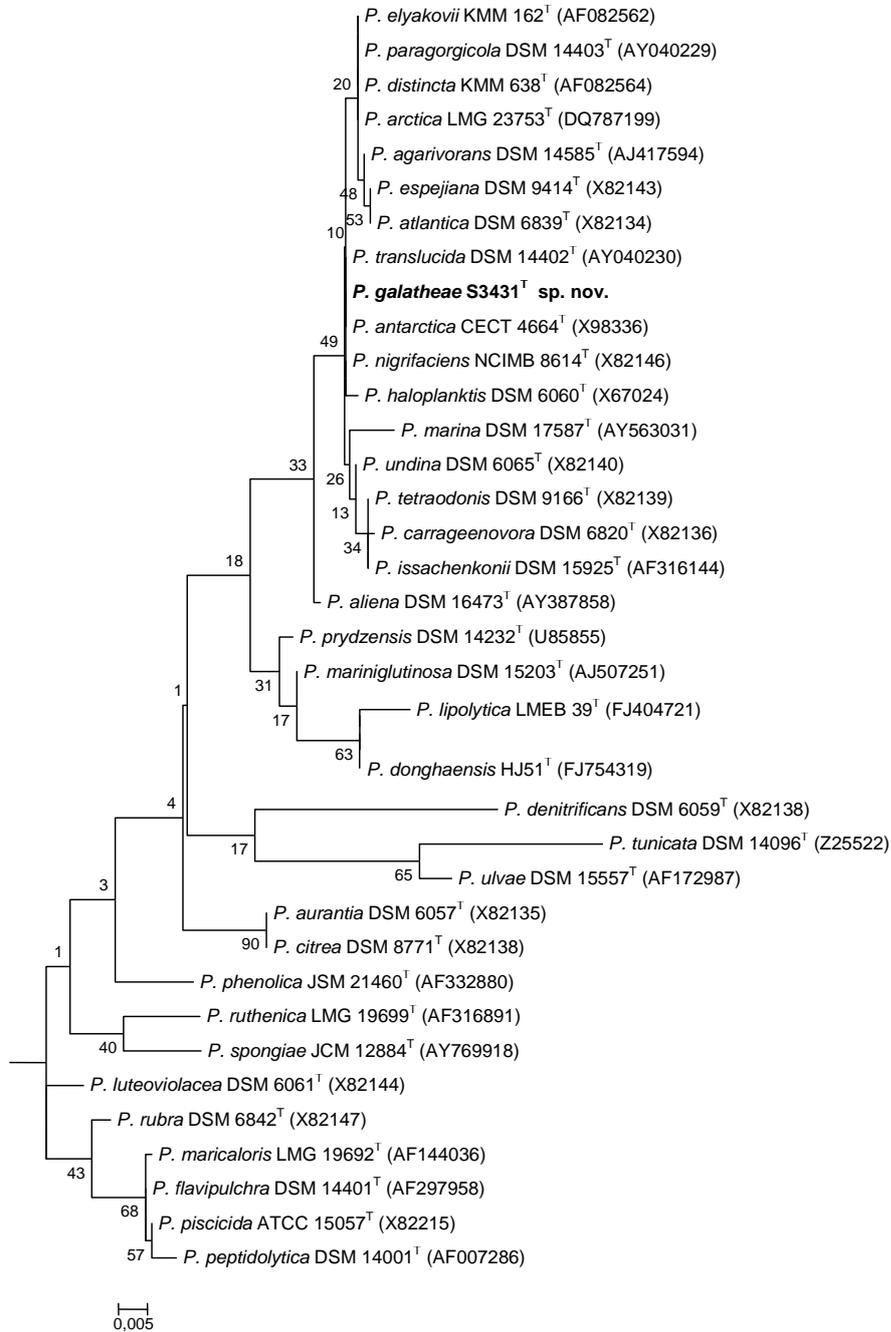
<i>Pseudoalteromonas</i> species	Per cent DNA:DNA similarity to strain S3431
<i>P. agarivorans</i> DSM 14585 <sup>T</sup>	28.2
<i>P. aliena</i> LMG 22059 <sup>T</sup>	44.5
<i>P. atlantica</i> NCOMB 301 <sup>T</sup>	26.9
<i>P. carrageenovora</i> DSM 6820 <sup>T</sup>	14.8
<i>P. distincta</i> CIP 105340 <sup>T</sup>	44.5
<i>P. elyakovii</i> LMG 14908 <sup>T</sup>	44.2
<i>P. espejiana</i> DSM 9414 <sup>T</sup>	48.0
<i>P. issachenkonii</i> LMG19697 <sup>T</sup>	33.6
<i>P. nigrifaciens</i> LMG 2227 <sup>T</sup>	15.4
<i>P. paragorgicola</i> LMG 19696 <sup>T</sup>	58.4
<i>P. tetraodonis</i> DSM 9166 <sup>T</sup>	11.3
<i>P. undina</i> LMG 2880 <sup>T</sup>	13.8
<i>P. antarctica</i> CECT4664 <sup>T</sup>	WIP

**Table 3:** Cellular fatty acid composition of *Pseudoalteromonas galathea* S3431<sup>T</sup>. Fatty acids constituting less than 0.5% of the total amount were not included. -, Not detected in significant amounts.

Fatty acid	S3431 <sup>T</sup>
C <sub>12:0</sub>	1.25
C <sub>14:0</sub>	0.51
C <sub>15:0</sub>	-
C <sub>16:0</sub>	20.43
C <sub>17:0</sub>	5.76
C <sub>18:0</sub>	1.14
C <sub>15:1</sub> ω8c	1.60
C <sub>17:1</sub> ω8c	10.85
C <sub>17:1</sub> ω6c	0.55
C <sub>18:1</sub> ω7c	7.77
C <sub>16:0</sub> iso	1.18
C <sub>17:0</sub> iso	0.60
C <sub>17:0</sub> anteiso	0.56
C <sub>10:0</sub> 3OH	1.07
C <sub>11:0</sub> 3OH	1.11
C <sub>12:0</sub> 3OH	5.15
Summed feature 1 (C <sub>15:0</sub> iso H/C <sub>13:0</sub> 3OH)	0.62
Summed feature 3 (C <sub>16:1</sub> ω7c/C <sub>16:1</sub> ω6c)	35.82
Other	4.03



**Supplementary figure 1:** Phylogenetic tree based on 16S rRNA gene sequences of *Pseudoalteromonas* type strains. The novel species *P. galathea* S3431 sp. nov. placed itself in the non-pigmented clade, near *P. translucida* DSM 14402<sup>T</sup>, *P. antarctica* CECT 4664<sup>T</sup>, *P. nigrifaciens* NCIMB 8614<sup>T</sup>, and *P. haloplanktis* DSM6060<sup>T</sup>. The 16S rRNA gene sequences were aligned in MEGA 5 and the phylogenetic reconstruction was based on the maximum likelihood method with the Kimura 2-parameter model, with a Gamma distribution model allowing invariant sites to model the evolutionary rate differences. Scale bar: 0,005 substitutions per nucleotide site.



## PAPER 4

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Vynne N, Månsson M, Nielsen KF, Milton D, Gram L (2012)

Production of a putatively novel acylated homoserine lactone-like quorum sensing activating molecule by *Pseudoalteromonas luteoviolacea* S4054

Manuscript in preparation.

1 Production of a putatively novel acylated homoserine lactone-like quorum sensing  
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3

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13 Keywords: Quorum sensing, *Pseudoalteromonas luteoviolacea*, antibiotics, lux,

14

15 Running title: AHL-like molecules in *Pseudoalteromonas luteoviolacea*

16 **Abstract**

17 The bioactive strain *Pseudoalteromonas luteoviolacea* S4054 produces antibiotics and compounds capable  
18 of eliciting a signal response in the quorum sensing monitor strains *Agrobacterium tumefaciens* pZLR4,  
19 *Chromobacterium violaceum* CV026, and *Escherichia coli* JM109 pSB401. Since quorum sensing in some  
20 cases control the production of antibiotics, we hypothesized that quorum sensing might be involved in  
21 control of antibiotic production by *P. luteoviolacea* S4054. Through shotgun cloning the *plul* gene was  
22 identified as the gene coding for production of the signaling compounds. *plul* showed homology to *luxI* AHL  
23 synthethases from *Vibrionaceae* and other Proteobacteria, but formed a monophyletic branch in  
24 phylogenetic analysis of *luxI* homologues indicating that *plul* encodes a novel AHL synthethase. Genome  
25 sequencing revealed that *plul* is located in an operon structure with downstream genes that may constitute  
26 a biosynthetic pathway potentially involved in biosynthesis of the potent antibiotic indolmycin.  
27 Immediately downsteam of the operon, the *luxR* homologue *pluR* was located on the opposite DNA strand.  
28 Chemical analysis of the signaling molecules revealed AHL-like traits, however it was not possible to identify  
29 the compounds using standard methods for AHL identification indicating that the signaling molecules might  
30 represent novel AHL-like compounds.

31

## 32 Introduction

33 Cell-density dependant signaling occurs widely among bacteria and is commonly termed quorum sensing  
34 (QS). QS allows a population of cells the benefits of coordinated gene expression, sensing the presence of  
35 other signal molecule producing microorganisms or possibly probing the local environmental conditions. A  
36 common QS system in the  $\gamma$ -Proteobacteria is the LuxI-LuxR system. This QS system was first described in  
37 *Vibrio fischeri* (8), where it regulates the expression of bioluminescence genes in a cell-density dependent  
38 manner. The signaling molecules of this system are *N*-acylated homoserine lactones (AHLs) and are  
39 produced by LuxI AHL synthethases (9). AHLs are produced continuously at low cell-densities AHLs, but as  
40 cell-density in the local environment increases, the concentration of AHLs reaches a threshold level and  
41 activates the QS regulated system through binding to the transcriptional regulator LuxR. This activates gene  
42 expression of the QS regulated phenotype, in the case of *V. fischeri* bioluminescence. In some bacteria,  
43 expression of the *luxI* gene is also increased leading to a strong autoinducing effect (25). Some non-AHL  
44 molecules such as diketopiperazines are capable of modulating the Lux-system to activate or repress the  
45 signaling response (11,15). Hence the QS regulated phenotypes may be influenced by AHLs or by other  
46 compounds.

47 For some bacteria, QS no doubt holds an important role in microbial community interactions through  
48 regulation of phenotypes such as biofilm formation, motility and production of antimicrobial compounds  
49 (10). In the marine environment, multiple bacterial species will be present on most surfaces and  
50 competition for space and nutrients is believed to play a major role in shaping the microbial community  
51 (14). The production of antibacterial compounds is one way for a bacterium to modulate the surrounding  
52 community and QS regulation of biosynthetic pathways for production of antibacterial compounds is well  
53 known (6,32). Recently production of the potent antibacterial compound tropodithietic acid was  
54 demonstrated to be QS controlled in *Phaeobacter gallaeciensis* (1), a member of the ecologically significant  
55 *Roseobacter* clade (2). Previous studies have indicated that AHLs are produced by *Pseudoalteromonas*

56 species associated with different microbial communities (17,18), however, few studies have addressed the  
57 actual regulatory role of the compounds. Violacein, which is a key metabolite of the *P. luteoviolacea* species  
58 and may protect against predation (22), is produced under QS regulation in both distant and closely related  
59 organisms such as *Chromobacterium violaceum* and *Pseudoalteromonas* sp. 520P1 (23,37). A LuxI-LuxR  
60 system in *P. luteoviolacea* could potentially regulate production of violacein, but also of other bioactive  
61 compounds such as pentabromopseudilin and indolmycin which we previously identified in a collection of  
62 *P. luteoviolacea* strains (20,35,35).

63 The purpose of the present study was to determine if QS regulatory systems could be involved in antibiotic  
64 production by *P. luteoviolacea*. We demonstrate that compounds inducing AHL-monitor bacteria are  
65 present in some but not all violacein producing *P. luteoviolacea* strains. In one AHL-monitor-inducing strain,  
66 *P. luteoviolacea* S4054, we identify a *luxI* gene homologue and we investigate the genomic context of this  
67 gene to identify a gene homologue to known *luxR* genes. We identify a putative novel biosynthetic pathway  
68 in an operon structure with the *luxI* gene, which is likely quorum sensing regulated. Thorough chemical  
69 analyses did, however, not lead to identification of any known AHL molecule and we conclude that the AHL-  
70 like compound(s) likely represent a novel AHL structure.

71

## 72 **Materials and methods**

73 **Strains, plasmids and media.** Thirteen *Pseudoalteromonas luteoviolacea* strains previously described (34)  
74 were included in this study. *P. luteoviolacea* strains were routinely cultured at 25 °C in a marine minimal  
75 medium (27) with 4 g/l mannose and 3 g/l casamino acids, unless otherwise mentioned. *Chromobacterium*  
76 *violaceum* CV026 was cultured in Luria broth (per litre: Bacto Tryptone, 10 g; Bacto yeast extract, 5 g; and  
77 sodium chloride, 5 g) at 25 °C with 20 µg/ml kanamycin (23). *Agrobacterium tumefaciens* pZLR4 was  
78 cultured in ABT medium (per litre: (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 0.4 g, Na<sub>2</sub>HPO<sub>4</sub> 0.6 g, KH<sub>2</sub>PO<sub>4</sub> 0.3 g, NaCl 0.3 g, 1 mM MgCl<sub>2</sub>,  
79 0.1 mM CaCl<sub>2</sub>, 0.01 mM FeCl<sub>3</sub>, 2.5 mg thiamin supplemented with 0.5% glucose and 0.5% cas-amino acids)

80 (4) supplemented with 20 µg/ml gentamicin. The bacterial strains and plasmids used for cloning  
81 procedures in this study are listed in table 1. *E. coli* strains were grown at 37 °C in Luria broth  
82 supplemented with carbenicillin 100 µg/ml or tetracyclin 10 µg/ml. All broth cultures were incubated with  
83 agitation, and supplemented with 1.2% agar as required.

84 **Bioassays for detection of potential QS activating molecules.** Well diffusion agar assays were used to  
85 screen for the presence of QS activating molecules in sterile filtered culture supernatants and ethyl acetate  
86 extracts. Ethyl acetate extracts were prepared as described for LC-MS analysis. To prepare the assays, 50 ml  
87 overnight culture of *C. violaceum* CV026 was added to 100 ml molten LB-agar at 46 °C, supplemented with  
88 20 µg/ml kanamycin (23), or 50 ml overnight culture of *A. tumefaciens* pZLR4 were added to 100 ml ABT-  
89 agar at 46 °C supplemented with 20 µg/ml gentamicin and 75 µg/ml X-gal (3). The solution was poured into  
90 petri dishes to form a 5 mm thick layer. Wells were cut after the agar had solidified and 50 µl sample was  
91 loaded per well. The assays were incubated at 25 °C and read after 24 and 48 h. Simple T-streaks against *C.*  
92 *violaceum* CV026 were used to screen *E. coli* clones for production of QS activating compounds (23).

93 The monitor strain *E. coli* JM109 pSB401 (40) was used as a heterologous host to express and detect  
94 potential QS activating molecules. The *E. coli* JM109 clones containing both the pSB401 plasmid and a  
95 pBluescript construct were plated on LB-agar with 50 µg/ml carbenicillin, 10 µg/ml tetracyclin, 20 µg/ml  
96 IPTG and 50 µg/ml X-gal, incubated overnight at 37 °C and screened for bioluminescence using a LAS-4000  
97 (Fujifilm Life Sciences, USA).

98 **Thin layer chromatography (TLC).** Ethyl acetate extracts or synthetic AHL standards were spotted onto a  
99 C<sub>18</sub> TLC plate (TLC aluminum sheets 20×20 cm<sup>2</sup>, RP-18 F<sub>254</sub>S, 1.05559. Merck 64271 Darmstadt, Germany)  
100 and the plates were developed in 10 ml 60:40 (v/v) methanol/water until the front reached the top (28).  
101 The TLC plate was dried and agar top-layers were prepared as for the well diffusion assay. The top layer of  
102 bacterium-containing agar was poured onto the TLC plate immediately following preparation, and  
103 incubated at 25 °C for 24h.

104 **PCR conditions, DNA techniques, DNA sequencing and bioinformatics.** PCR was performed as previously  
105 described (5,24). Unless otherwise stated, all conditions for the various DNA techniques were as described  
106 by (30). Reaction conditions for DNA-restriction enzymes were as suggested by the manufacturers. DNA  
107 sequencing of the unknown DNA regions obtained from shotgun cloning was performed by primer walking  
108 in two directions from known regions of DNA sequence using the primers listed in table 2. Sequence data  
109 were processed and assembled in CLC-bio DNA workbench, and MEGA 5 was used for sequence alignment  
110 and phylogenetic reconstruction.

111 **Shotgun cloning to identify genes involved in QS.** Genomic DNA was purified from *P. luteoviolacea* S4054  
112 using the QIAGEN Genomic-Tip/100 kit. The genomic DNA was digested with EcoRI or BamHI and ligated  
113 into the corresponding pBluescript digest. The shotgun library obtained in pBluescript was transformed into  
114 chemically competent *E. coli* JM109 pSB401, incubated overnight at 37 °C and screened for luminescence.  
115 Luminescent clones were tested for induction of violacein production in *C. violaceum* CV026 by T-streaks,  
116 and the pBluescript vector from positive clones was cloned by transformation into chemically competent *E.*  
117 *coli* DH5- $\alpha$   $\lambda$ pir, plated on LB-agar with 50  $\mu$ g/ml carbenicillin and incubated overnight at 37 °C. The  
118 resulting clones were verified for activity in the *C. violaceum* CV026 bioassay and the pBluescript DNA insert  
119 was sequenced using a primer walking method.

120 **LC-MS and LC-MS/MS analyses.** *P. luteoviolacea* S4054, *E. coli* NV5, and *E. coli* NV1 were grown in MMM  
121 for 3 days at 25°C and the cultures were extracted with equal volumes of ethyl acetate, centrifuged, and  
122 the ethyl acetate phase was evaporated under N<sub>2</sub> to dryness. Samples were redissolved in 500  $\mu$ l MeOH  
123 and analysed by LC-MS. LC-MS analyses were performed on an Agilent 1100 System equipped with a  
124 UV/VIS photo diode array detector (scanning 200–600 nm). Separation was done on a 100 mm $\times$ 2 mm i.d., 3  
125  $\mu$ m Gemini C6-phenyl column (Phenomenex, Torrance, CA, USA), running at 40°C using gradient (15-100%  
126 over 20 minutes) of water (H<sub>2</sub>O) and acetonitrile (MeCN); both buffered with 50  $\mu$ l/l trifluoroacetic acid) at  
127 a flow of 300  $\mu$ l/min. LC-MS/MS analyses were performed on a maXis quadrupole time of flight mass

128 spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray (ESI) ion source. The MS  
129 was connected to an Ultimate 3000 UHPLC system (Dionex, Sunnyvale, CA) equipped with a diode-array  
130 detector. Separation was performed at 40 °C on a 150 mm × 2.1 mm ID, 2.6 μm Kinetex C18 column  
131 (Phenomenex) using a linear water/MeCN (both buffered with 20 mM formic acid) gradient starting from  
132 15% MeCN and increased to 100% in 13 min at a flow of 0.4 mL min<sup>-1</sup>. Experiments were performed in ESI+  
133 with a data acquisition range of *m/z* 100–1200 with collision energy of 40 V. The MS was calibrated using  
134 sodium formate automatically infused prior to each analytical run.

135 **Purification and structure elucidation of the QS activating molecule.** Preparative TLC was performed on  
136 C18 plates (20 x 20 cm, Analtech). On each plate, approximately 20 mg of crude extract was loaded in 500  
137 μL 50/50 (v/v) MeOH/H<sub>2</sub>O with a solvent streaker. Plates were developed in 60% MeOH in water. To  
138 visualize the active compounds, the plates were overlaid with agar containing *C. violaceum* CV026 or *A.*  
139 *tumefaciens* pZLR4 as described above but limited to a 3 cm wide band at the left and right edges.

140 Three different solid phase extraction columns were attempted on a small scale (2 mg crude extract):  
141 Strata-X (30 mg/1 mL, Phenomenex), Strata-NH<sub>2</sub> (100 mg/1 mL, Phenomenex), and diol (100 mg/1 mL,  
142 Phenomenex). From all three columns, five fractions were collected and tested in the QS assays. From  
143 Strata-X: 100% H<sub>2</sub>O, 25% MeOH in H<sub>2</sub>O, 40% MeOH, 60% MeOH, and 100% MeOH. From Strata-NH<sub>2</sub>: 95%  
144 MeCN in H<sub>2</sub>O, 60% MeCN, 40% MeCN, 25% MeCN, and 100% H<sub>2</sub>O. From diol: 100% heptane, 100%  
145 dichloromethane, 100% EtOAc, 50% EtOAc in MeOH, and 100% MeOH. Fractions were evaporated under N<sub>2</sub>  
146 and redissolved in 100% MeOH immediately before biotesting.

147 An 8 L culture of *P. luteoviolacea* S4054 was grown in Marine Broth 2216 (Difco, USA). On day 3, a mixture  
148 (50/50) of sterile XAD-7 (Sigma-Aldrich, St. Louis, MO) and Diaion HP20SS resin (Sigma-Aldrich) was added  
149 to the broth (12 g of resin L<sup>-1</sup>). After 24 h the resin was filtered off and washed with water (2 x 2 L),  
150 followed by extraction with methanol/water (50/50 v/v; 800 mL) (24h) and methanol (2 x 800 mL) (24 h).  
151 All organic extracts were pooled and dried completely to give a crude extract (2.0712 g). Violacein and  
152 indolmycin were quantitatively removed from the extract by cation-exchange as previously described (20).

153 The remaining extract was subjected to anion-exchange. The crude extract (640 mg) was re-dissolved in  
154 methanol/water (50/50 v/v; 5 ml) and mixed with 3 g Strata-SAX (Phenomenex, Torrance, CA) and dried  
155 before packing into a 100 g SNAP column (Biotage, Uppsala, Sweden). Using an Isolera flash purification  
156 system (Biotage), the column was equilibrated (5 mL/min) upside-down with water + ammonium hydroxide  
157 (2%). The column was then turned and washed with methanol with ammonium hydroxide (2%) (fraction 1).  
158 The column was then stripped with methanol with formic acid (1%) and washed until no colour remained  
159 (fraction 2). Fraction 1, enriched with QS activity, was redissolved in EtOAc and absorbed onto xx g Isolute  
160 diol (Biotage) and added to a 100 g SNAP column with pure diol (10 g). A total of 12 fractions were collected  
161 ranging from heptane, dichloromethane, EtOAc to pure MeOH (5 mL/min). The fractions with QS activity  
162 (100 % dichloromethane to 20% dicholormethane) were pooled and further separated on a Luna II C18  
163 column (250 × 10 mm, 5 µm) (Phenomenex) using a 10-90% MeCN/H<sub>2</sub>O gradient (buffered with 20 mM FA,  
164 flow rate 5 mL/min) over 20 minutes on a Gilson 322 liquid chromatograph with a 215 liquid  
165 handler/injector (BioLab, Risskov, Denmark). This yielded four fractions with QS activity. NMR spectra were  
166 recorded on a Varian Unity Inova 500 MHz spectrometer equipped with a 5 mm probe using standard pulse  
167 sequences. The signals of the solvent were used as internal references ( $\delta$ H 4.80 for D<sub>2</sub>O).

168 **Stability test of AHL-like compounds.** The lactone ring of classic AHLs opens under alkaline conditions,  
169 leading to a loss of QS inducing activity. To test the AHL-like nature of the compounds obtained from  
170 chemical fractionation, the compounds and a mix of synthetic AHLs were suspended in solutions of pH 2,  
171 pH 7, and pH 11 for two hours, after which samples were tested in well diffusion agar assays against the  
172 monitor strain *A. tumefaciens* pZLR4.

173 **Genome mining for *luxI* – *luxR* gene pairs and quorum sensing controlled gene clusters.** A permanent  
174 draft genome of *P. luteoviolacea* was obtained by sequencing and assembly as described elsewhere (33).  
175 The genome sequence was submitted to the IMG ER pipeline for annotation and visualization (21). The  
176 nucleotide sequence obtained from cloning experiments was identified and the neighboring gene clusters  
177 investigated. *luxR* homologue genes were identified by a search for genes with the Pfam 03472 autoinducer

178 binding domain or genes in COG2771 . The resulting genes were submitted to BLASTX and LuxR to identify  
179 LuxR homologues. The gene cluster downstream of the *luxI* homologue gene was investigated for its  
180 putative biological function through analysis of the annotations and BLAST.

181

## 182 **Results and discussion**

### 183 **Characterization and production of AHL-like molecules by *Pseudoalteromonas luteoviolacea* strains.**

184 Thirteen *Pseudoalteromonas luteoviolacea* strains were cultured overnight in MMM and sterile filtered  
185 supernatants were tested in well assays using *C. violaceum* CV026 or *A. tumefaciens* pZLR4 monitor strains.  
186 Three strains (S4047-1, S4054, and CPMOR-1) activated both monitor strains indicating the presence of  
187 AHL-like compounds in the growth supernatants. Ten strains did not activate either monitor strain, but  
188 were previously shown to produce violacein. Hence, violacein production in *P. luteoviolacea* is not  
189 universally regulated by molecules that elicit a response in traditional QS monitor strains. We previously  
190 showed that the three strains are closely related produce indolmycin, an antibiotic compound not  
191 produced by the remaining 10 *P. luteoviolacea* strains (34), hence it was tempting to speculate that  
192 indolmycin production could be QS regulated. Organic extracts of overnight cultures of the QS-activating  
193 strains were prepared and loaded on a TLC plate, subsequently overlaid with *A. tumefaciens* pZLR4 (figure  
194 1). Figure 1 shows the presence of at least three putative AHL-like compounds in the culture supernatant of  
195 strain S4054, which was chosen for further studies of the potential QS system.

196 **Cloning and characterization of a *luxI* homologue from *P. luteoviolacea* S4054.** To elucidate the genetics  
197 of the putative QS system in *P. luteoviolacea* S4054 a shotgun cloning approach was applied, in which a  
198 shotgun library was screened for its ability to induce luminescence in *E. coli* JM109 pSB401 when expressed  
199 in the same host. Twenty three luminescent clones were isolated and tested against the *C. violaceum*  
200 CV026 monitor strain. Clones NV-5 and NV-17 elicited the strongest response based on the intensity of  
201 violacein production in CV026 and in TLC bioassays a pattern similar to the wild type was observed. Hence,

202 the DNA fragment cloned from S4054 encodes a gene which is expressed in *E. coli* DH5- $\alpha$   $\lambda$ pir and enables  
203 synthesis of AHL-like compounds. A restriction pattern analysis was carried out on the insert DNA  
204 fragments from positive clones which indicated that NV-5 and NV-17 clones contained the same DNA  
205 fragment, with an additional fragment in NV-17 (data not shown). Since NV-5 produced the strongest  
206 response in CV026 and contained the shortest DNA insert, this construct was chosen for sequencing based  
207 on a primer walking strategy. In the resulting nucleotide sequence the gene *plul* with homology to various  
208 *luxI* genes was identified using BLAST. The 20 best BLASTX hits were used to produce the phylogenetic tree  
209 in figure 2, which placed Plul on a monophyletic branch adjacent to LuxI proteins from primarily  
210 *Vibrionaceae*, which are known to synthesize AHL molecules (9). Hence, molecular and bioinformatics  
211 analyses provided evidence that *plul* encodes a novel LuxI homologue AHL-synthetase involved in the  
212 production of AHL-like molecules by *P. luteoviolacea* S4054.

213 **Analysis of the genomic context of *plul*.** To facilitate analysis of the genomic context of the *plul* gene whole  
214 genome sequencing was done (33). The genome confirmed the *plul* sequence obtained by cloning and  
215 subsequent primer walking. Upstream of *plul* a palindromic sequence resembling a Lux-box was identified.  
216 In canonic LuxIR systems the Lux-box is targeted by the LuxR protein for DNA binding suggesting that the  
217 downstream genes are QS regulated. *plul* was situated in an operon structure (figure 3) and immediately  
218 following the operon, a *luxR* homologue *pluR* was identified on the opposite DNA strand. The genes *plul*  
219 and *pluR* likely form a QS system for regulation of gene expression. The putative operon structure very  
220 likely contained genes regulated by this PluIR system in a genomic organization parallel to the *V. fischeri*  
221 *luxI-CDABEG* genes (8). Annotation of the genes contained in the operon structure showed functions such  
222 as aminotransferases and a methyltransferase which are included in the biosynthetic pathway of the  
223 antibiotic indolmycin (16), however as the genes involved in biosynthesis of indolmycin are not known,  
224 further studies are required to determine the actual product of the putative *pluIR* regulated operon. The  
225 presence of a tryptophanyl tRNA synthetase in the operon also suggests a link to indolmycin, which is  
226 tryptophan derived and selectively targets tryptophanyl tRNA to exert its antibacterial activity (38).

227 **Chemical analysis of AHL-like compounds from *P. luteoviolacea* S4054.** The supernatant was analyzed for  
228 the presence of AHL-like molecules in the TLC assay with *Agrobacterium* and *Chromobacterium*. None of  
229 the signals were present in the blank medium. The strong response in the *A. tumefaciens* pZLR4 monitor  
230 strain coupled with a weaker response in *C. violaceum* CV026 suggests that the QS activating molecules  
231 share characteristics with 3-oxo substituted AHLs (3). When subjected to alkaline conditions, the synthetic  
232 AHL standards and the QS activating compounds isolated from strain S4054 lost the ability to elicit a  
233 response in the QS monitor strain *A. tumefaciens* pZLR4, as would be expected from AHLs (42).

234 Ethyl acetate extracts and supernatants of cultures of s4054 were investigated for the presence of AHLs  
235 using validated LC-HRMS methods (36). No peaks matched the retention time or accurate mass of  
236 commonly found AHL compounds or any analogues reported in literature (19). LC-MS/MS experiments (26)  
237 with in-source fragmentation (40 V) were performed in order to confirm the presence of potential novel  
238 AHL analogues. Blank medium spiked with AHL standards was used as positive control to ensure that there  
239 was no ion suppression. However, the characteristic fragment of 102 Da corresponding to the lactone  
240 moiety of the AHLs could not be observed in any of the *P. luteoviolacea* extracts. With the potential of a  
241 completely novel QS compound, the metabolite profiles of s4054 and the *E. coli* clone NV5 were compared  
242 as common peaks are likely candidates for the QS compound(s). The *E. coli* clone NV1 containing the  
243 pBluescript vector without the *luxI* insert was used as negative control. However, no shared compounds  
244 were detected between S4054 and clone NV5, suggesting that the AHL molecules were present in very low  
245 amounts or did not perform well in the chromatography. S4054 and NV5 were subjected to preparative  
246 reverse phase C18 TLC in order to investigate more pure fractions of the QS compound, yet it was not  
247 possible to obtain sufficient resolution. Likewise, it was not possible to obtain resolution of the QS activity  
248 when subjecting the crude extracts to different solid phase extraction columns (C18, diol, amino) with a  
249 finer particle size. As the QS compound might be present in concentrations below the detection limits of  
250 our instruments, an 8 L culture was prepared and subjected to cation- and anion-exchange to remove  
251 charged compounds. As expected for AHL-like compounds, the activity was recovered from the neutral

252 fraction. Subsequent purification was done on diol and C18 to obtain orthogonal separation of the  
253 compounds. This led to four fractions with QS activity. However, it was not possible to deduce an accurate  
254 mass from LC-MS analyses. The active fractions were analysed by NMR, which revealed the presence of an  
255 NH-proton and a large aliphatic moiety consistent with an AHL; however, the amounts obtained were  
256 insufficient for detailed structural analysis. Overall, NMR and chromatographic behavior suggest that we  
257 are dealing with an AHL-like molecule, potentially with a modification in the homoserine lactone or with  
258 multiple oxo/hydroxyl-groups causing tailing which makes it difficult to detect on columns traditionally  
259 used for AHL purification (36).

260

261

262 **Conclusion.** *P. luteoviolacea* S4054 produced molecules that elicited QS responses in the monitor strains  
263 *C.violaceum* CV026, *A. tumefaciens* pZLR4, and *E. coli* JM109 pSB401. This, in addition to genomic evidence  
264 and indications from chemical analyses, provided strong evidence for the AHL-like nature of these  
265 molecules, however we were unable to elucidate the complete structure of the active compounds. The *plul*  
266 gene forms a monophyletic cluster in a phylogenetic analysis of *luxI* homologues which indicates that it  
267 might represent a novel family of LuxI synthetase homologues. The *plul* gene was located in an operon  
268 structure with a potential biosynthetic pathway downstream of *plul*. This pathway was not related to the  
269 violacein pathway, which appears to not be quorum sensing regulated in *P. luteoviolacea*, but some gene  
270 functions corresponded to known biosynthetic steps in the biosynthesis of the potent antibiotic indolmycin.  
271 Quorum sensing regulation of antibiotic production is potentially an important modulating factor in  
272 microbial communities, and further work is warranted to elucidate the mechanisms of the *P. luteoviolacea*  
273 S4054 QS system and its *in situ* function.

274

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277 expedition under the auspices of the Danish Expedition Foundation. This is Galathea 3 contribution no.  
278 PXXXXXXX (to be added if/when accepted).

279

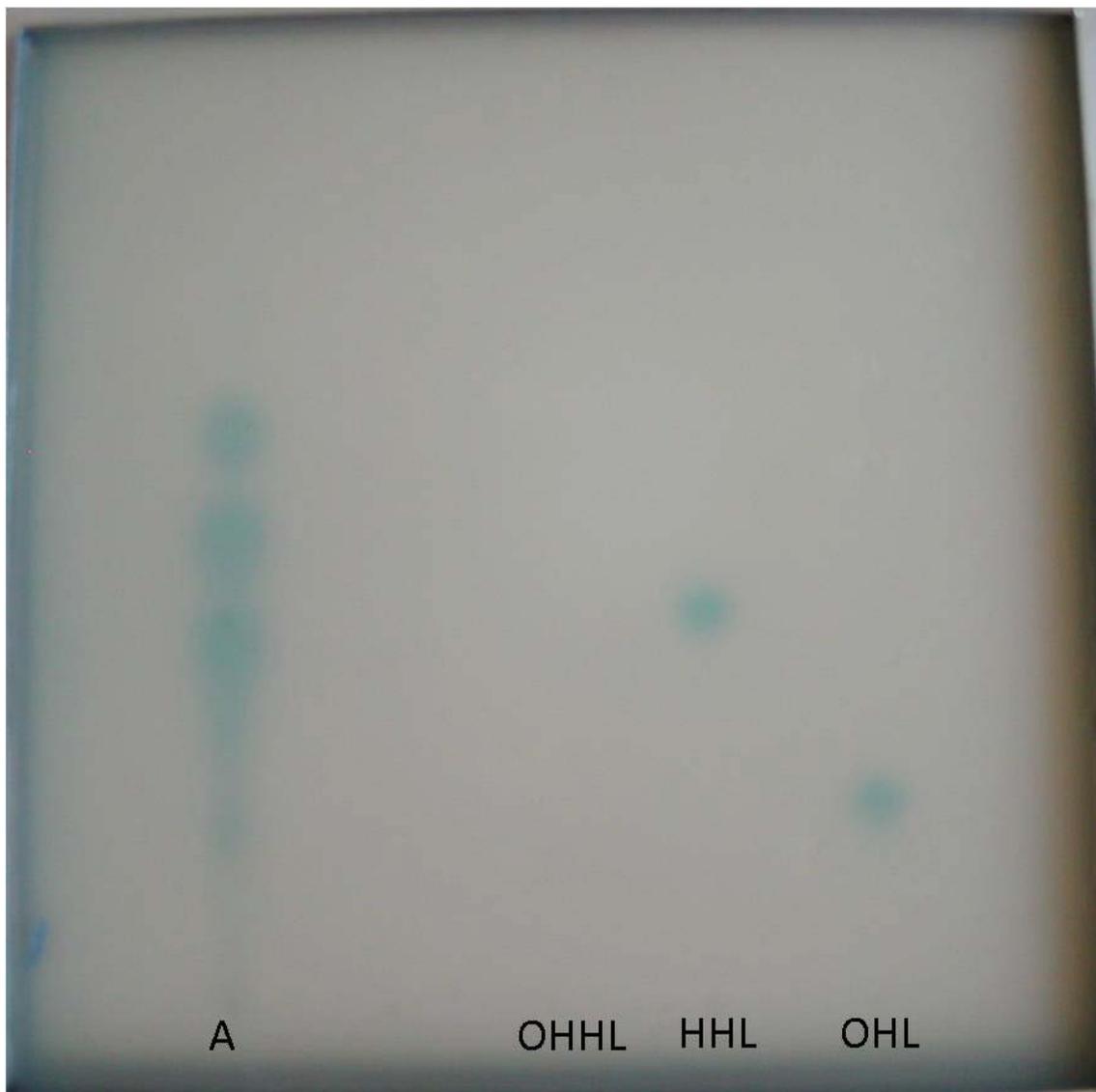


Figure 1: TLC of extract from *P. luteoviolacea* S4054. A: 10 µl ethyl acetate extract from *P. luteoviolacea* overnight culture in MMM. At least three spots are visible in lane A, indicative of three or more AHL-like compounds in the sample. The amounts of synthetic AHLs loaded as standards were: OHHL 10 pM, HHL 3 nM, and OHL 3.8 pM.

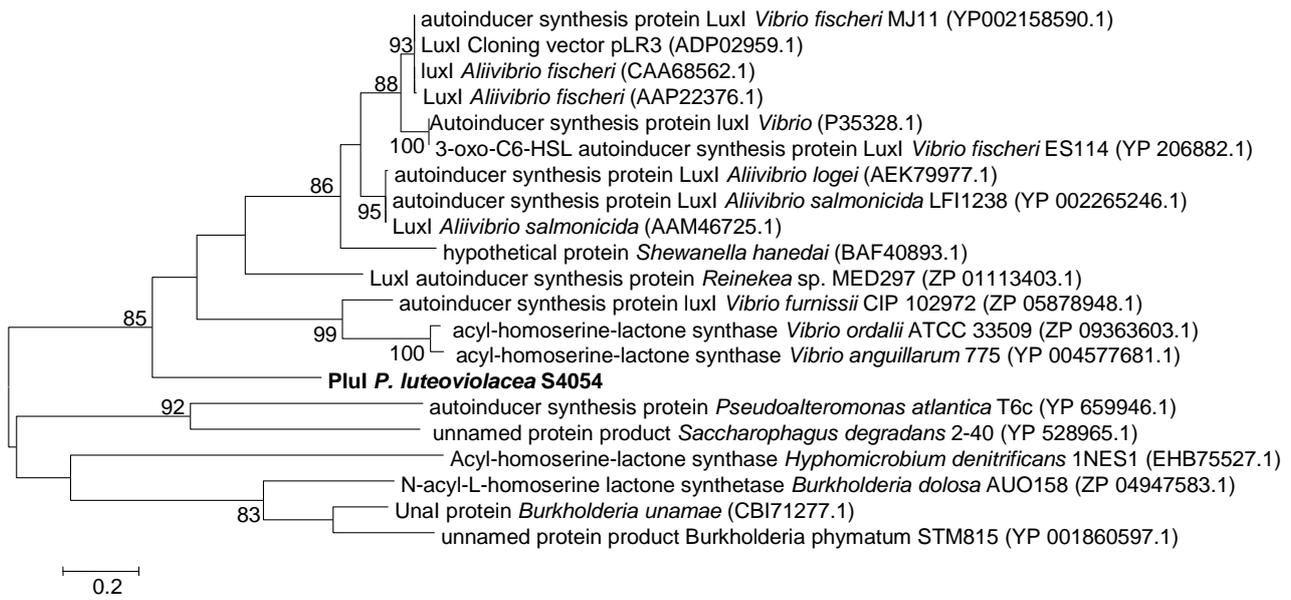


Figure 2: Phylogenetic tree of the top 20 BLASTX hits for the *plul* gene. The tree is based on a multiple sequence alignment of translated nucleotide sequences calculated in MEGA5 (31), which was also used to infer the phylogeny using the maximum likelihood method based on the Whelan and Goldman model (39). The rigidity of the tree was tested using 100 bootstrap replications as indicated for nodes supported 70% or higher. Plul forms a monophyletic branch adjacent to LuxI proteins from mostly *Vibrionaceae* and far removed from the *P. atlantica* protein. This supports the hypothesis that Plul represents a novel AHL synthetase. Scale bar: substitutions per site.

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282



Figure 3: Genomic organization of QS related elements in *P. luteoviolacea* S4054 and a putatively QS regulated operon. Predicted functions: Dark blue: *pluI*, red: aminotransferase, yellow: dehydrogenase, green: methyltransferase, grey: hypothetical protein, purple: coenzyme F390 synthetase, pink: methylase, brown: lyase, olive: tryptophanyl t-RNA synthetase, light blue: *pluR*. Upstream of *pluI* a palindromic sequence reminiscent of a *lux*-box was identified (not shown).

Table 1: Bacterial strains and plasmids used in this study.

Strain or plasmids	Genotype or relevant markers	Source
<b><i>Pseudoalteromonas luteoviolacea</i></b>		
DSM 6061 (T)	Type strain, wild type	DSMZ
S2607	Wild type	(13)
S4047-1	Wild type	(13)
S4054	Wild type	(13)
S4060-1	Wild type	(13)
CPMOR-1	Wild type	(12)
CPMOR-2	Wild type	(12)
2ta16	Wild type	(29)
H33	Wild type	T. Harder, unpublished
H33S	Wild type	T. Harder, unpublished
NCIMB 1942	Wild type	NCIMB
NCIMB 1944	Wild type	NCIMB
NCIMB 2035	Wild type	NCIMB
<b><i>Chromobacterium violaceum</i></b>		
<b>CV026</b>		
<b><i>Agrobacterium tumefaciens</i></b>		
<b>pZLR4</b>		
<b><i>Escherichia coli</i></b>		
DH5 $\alpha$ - $\lambda$ <i>pir</i>	F <sup>-</sup> $\Phi$ 80 <i>dlacZ</i> $\Delta$ M15 $\Delta$ ( <i>lacZYA-argF</i> <i>U169 deoR supE44 hsdR17 recA1</i> <i>endA1 gyrA96 thi-1 relA1</i>	(7)
JM109	<i>recA1 endA1 gyrA96 thi hsdR17</i> <i>supE44 relA1</i> $\Delta$ ( <i>lac-</i> <i>proAB</i> ) <i>mcrA</i> [F' <i>traD36 proAB</i> <i>lacI<sup>q</sup>lacZ</i> $\Delta$ M	(41)
<b>Plasmids</b>		
pBluescript	Ap <sup>r</sup> ; ColE1 origin	Stratagene
pSB401	Tc <sup>r</sup> ; p15A origin	(40)

284

285

Table 2: Primers used for sequencing.

Primer	Sequence 5'-3'
T3	AAT TAA CCC TCA CTA AAG GG
T7	GTA ATA CGA CTC ACT ATA GGG C
NV5-1	TCG GGT GAG AAT ATT
NV5-2	ACG TAA TCA ATG CCT
NV5-3	AAA ACA TGG CCA ACC
NV5-4	TTG AAG AGT TTG CGT
NV5-5	TAT CGC GGC AAA TCG
NV5-6	TGT TAC TTA ATG AAG
NV5-7	CTT CTT GCC AAT AGC
NV5-8	AGC TCT ATC CCT GAT
NV5-9	GTA TTG GCG CGT TCA
NV5-10	CTA CGT TAC GTT TAC
NV5-11	GCT TCA TTG TAA TAC
NV5-12	TGC AGT AGA TAA GCA
NV5-13	CGA GTT TAA AGC CGT
NV5-14	GTG AGC TGT TCG CAG

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Draft genome sequence of *Pseudoalteromonas luteoviolacea* S4054, an indolmycin  
producing marine bacterium

Manuscript in preparation.

1 **Draft genome sequence of *Pseudoalteromonas luteoviolacea* S4054, an indolmycin producing**  
2 **marine bacterium**

3  
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12  
13 **Abstract:** The ability to produce antibacterial compounds is wide spread among members of the  
14 genus *Pseudoalteromonas*. Here we present the 6.08-Mb draft genome sequence of *P. luteoviolacea*  
15 S4054, which produces the antibiotics violacein and indolmycin, and investigate the potential for  
16 further discoveries of bioactive compounds.

17  
18 Marine bacteria are widely investigated as a potential source of novel molecules with  
19 pharmacologically relevant activities. Several species of the genus *Pseudoalteromonas* produce  
20 antibacterial, antifouling or cytotoxic compounds (9) (1), however the ecological role of these  
21 compounds is not yet fully understood. Often, the bioactive *Pseudoalteromonas* have been isolated  
22 from the surfaces of marine eukaryotes and their bioactivity may be part of a symbiotic relationship  
23 (2). *P. luteoviolacea* S4054 was isolated from a seaweed sample in the Pacific Ocean and it produces  
24 the antibacterial compounds violacein and indolmycin (9) (7). Violacein is produced by different  
25 bacterial species, while to our knowledge indolmycin has not previously been isolated from a marine  
26 bacterium. The biosynthetic pathways responsible for production of these compounds are candidates  
27 for horizontal gene transfer events in S4054, however, only the violacein pathway is described on a  
28 genetic level, enabling identification through homology searches.

29  
30 Genomic DNA was obtained by successive phenol-chloroform purification steps. Mated paired-end  
31 library preparation, Illumina Hi Seq 2000 sequencing runs and initial data processing were done by  
32 BGI (Beijing, China). After quality filtering and trimming, the short reads were assembled and gaps  
33 filled using SOAPdenovo (6). This resulted in 126 contigs at a depth of 30.8-fold genome coverage  
34 and a total length of 6,076,381 bp. The draft genome was uploaded to the IMG/ER pipeline for gene  
35 annotation. A total of 5,268 genes were annotated, 5,121 of which were protein encoding. One  
36 hundred and twenty six genes fell into COG categories in secondary metabolism, with 30 genes in  
37 COG1020 (non-ribosomal peptide synthase modules and related proteins) and 20 genes in COG1028  
38 (dehydrogenases). Ten genes encoded proteins involved in PKS biosynthesis. BLASTP homology  
39 searches identified 5 genes encoding proteins VioA, B, C, D and E which form the violacein  
40 biosynthetic pathway (5). Upstream of the VioA-E genes, a gene encoding a multi-antimicrobial and

1 toxic compound extrusion pump was located, similar to the organization found in the  
2 *Pseudoalteromonas tunicata* D2 genome (8). In addition to the violacein pathway, six biosynthetic  
3 pathways were identified containing clusters of potential polyketide synthase or nonribosomal peptide  
4 synthesis coding genes. Genes encoding surface attachment related functions such as curli, type IV  
5 pili and P pili were identified, suggesting a surface associated lifestyle. The genome contained a  
6 necrosis inducing protein NPP1 homologue indicating a potential for plant pathogenicity (3). In  
7 addition, the genome had potential for quorum sensing regulated processes based on the presence of  
8 one pair of *luxI* - *luxR* genes (4).

9  
10 The data presented here provides further insight on the genetic potential for production of bioactive  
11 secondary metabolites within the pseudoalteromonads, and we believe it may advance studies on the  
12 ecology of these organisms.

13  
14 **Nucleotide accession number** The draft genome sequence for *P. luteoviolacea* S4054 is deposited  
15 in GenBank under the accession number XXXXXXXXXXXXXXXXX.

16  
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