Università degli Studi di Catania

Dipartimento in Scienze Biologiche, Geologiche ed Ambientali

Dottorato di Ricerca in Biotecnologie
(XXV ciclo: 2009-2013)

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Biological activity and regulation of cyclic lipopeptide production in *Pseudomonas corrugata* and *Pseudomonas mediterranea*

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List of Abbreviations

3-oxo-C_6-AHL	N-3-oxo-hexanoyl-L-Homoserine-lactone
3-oxo-C_8AHL	N-3-oxo-octanoyl-L-Homoserine lactone
3-oxo-C_{10}-AHL	N-3-oxo-octanoyl-L-Homoserine lactone
C_4-AHL	N-butyryl-L-Homoserine lactone
C_6-AHL	N-hexanoyl-L-Homoserine-lactone
C_7-AHL	N-heptanoyl-L-Homoserine lactone
C_8-AHL	N-octanoyl-L-Homoserine lactone
CH_3CN	Acetonitrile
CM-A	Cormycin-A
CPs	Corpeptins
BCA	Biological Control Agent
CFBP	Collection Francaise de Bacteries Phytopathogenes, Angers, France.
CLP	Cyclic Lipopeptides (i.e. LDP)
AHL	Acyl-homoserine-lactones
Amp	Ampicillin
BLAST	Basic Local Alignment Search Tool
BLASTP	Protein BLAST
bp	Base pairs
CLP	Cyclic lipopeptide
Cm	chloramphenicol
DI	Disease index
FPs	Fuscopeptins
Gm	Gentamycin
HCN	Hydrogen cyanide
HTH	Helix turn helix
IPTG	Isopropyl β-D-Thiogalactopyranoside
Kb	Kilobases
Km	Kanamycin
LDP	Lipodepsipeptide (i.e. CLP)

MALDI-TOF	Time of Light Matrix-assisted laser desorption/ionization
m/z	Mass to charge ratio
NRPS	Non ribosomal peptide synthetase
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
QS	Quorum Sensing
Rpm	Revolutions per minute
SPs	Syringopeptins
SR-E	Syringomycin-E
ST	Syringotoxin
TLC	Thin Layer Chromatography
TPN	Tomato pith necrosis
V/V	Volume to volume
X-Gal	5-Bromo-4-Chloro-3-Indolyl β-D-galactopyranoside

Summary

Pseudomonas corrugata and *P. mediterranea*, are two closely related bacteria both causal agents of tomato pith necrosis and recently evaluated as biocontrol agents and producers of industrially-promising biomolecules. The interest of our work focused on cyclic lipopeptides (CLPs) that are surface active molecules with antibacterial, antifungal and cytotoxic properties. *P. corrugata* is known to produce two kinds of cyclic lipopeptides, corpeptins and cormycin A. Investigations on *P. corrugata* strain CFBP5454 quorum sensing (QS) system helped to postulate its role on virulence mechanism mediated by CLPs during detrimental interaction with tomato plants. On the contrary no information was available on *P. mediterranea*/tomato molecular interaction. Both species are able to induce on tomato undistinguishable symptoms thus we thoughts that they likely share common virulence mechanisms. Starting from these premises we demonstrated that *P. mediterranea* CFBP 5447 harbours one system designated PmeI/R that is highly homologous to the PcoI/R system of *P. corrugata* and that virulence on tomatoes is affected by inactivation of QS genes in both species although with some differences. A LuxR regulator RfiA encoded by *rfiA* cotranscribed both with *pcoI* and *pmeI* was also demonstrated to be pivotal for virulence. The MALDI-TOF MS spectra of bacterial cell-free culture filtrates showed that *P. corrugata* CFBP5454 produces both corpeptins and cormycin and for the first time these CLPs were also found in a *P. mediterranea* strain which spectrum also showed a putatively unidentified bioactive compound. Mutational analysis revealed that *P. mediterranea* CFBP 5447 PmeI- and PmeR-, *P. corrugata* CFBP 5454 PcoR- and both RfiA-*,* were impaired in CLP production but also showed a drastic reduction of virulence when inoculated in tomato plants demonstrating their pivotal role. These results also evidence that the QS is involved in CLP production and all the correlated phenotypes (colony morphology, biosurfactant activity) via the RfiA transcriptional regulator.

In the *P. corrugata* CFBP 5454 cosmid insert sequence from which the AHL QS genes were identified we describes three genes *crpC*, *crpD*, *crpE.* Bioinformatics and mutational analysis of *crpC* and *crpDE* demonstrated they are involved in the production and secretion of corpeptins since the derivative mutant strain cell-free culture filtrates showed only the peaks of cormycin in their MALDI-TOF MS spectra. Moreover, pathogenicity tests revealed that these long chain CLPs are important for *P. corrugata* virulence. Using real-time PCR we

also ascertained that the regulators PcoR and RfiA are involved in corpeptins production/secretion.

P. corrugata CFBP 5454 and *P. mediterranea* CFBP5447 produced *in vitro* diffusible compounds with antimicrobial properties which inhibited the growth of plant pathogenic fungi and bacteria, as well as antifungal volatile compounds. Antagonistic activity of QS mutants didn't different from that of their respective parent strains whereas antifungal activity was completely abolished in the *rfiA*- mutants. Since both the *pcoR*- (and *pmeR*-) and *rfiA*- mutants did not produce CLPs the production of one or more additional antimicrobial compound is suggested. *In vivo* experiment showed the good biocontrol activity of *P. corrugata* CFBP 5454 but also that the QS and *rfiA* mutants are still able to reduce the disease incidence in the *in vivo* investigated pathosystems. *P. corrugata* CFBP 5454 draft genome sequence helped in identifying a biosynthetic cluster *hcnABC* coding for the enzyme HCN synthase involved in this volatile compound biosynthesis. *P. corrugata hcnA* and *hcnC* knockout mutants failed to produce HCN and showed a reduced antimicrobial activity against *Botrytis cinerea*. Cyanogenesis seems to be not regulated either by AHL-QS or by RfiA because mutants still retained the ability to produce HCN.

1 . Introduction

1.1. Plant-associated *Pseudomonas*

1.1.1. The genus *Pseudomonas*

The name *Pseudomonas* came to mind to professor Migula at the end of the nineteenth century.This description of the new genus, was short, inaccurate and although he never clarified the etymology of the word *Pseudomonas* in any of his writings, suggested to later taxonomists a direct derivation from the Greek *monas* or *unit.* Thus, in the 7th edition of Bergey's Manual of Determinative Bacteriology of 1957, was found the definition of *Pseudomonas* as 'Gr. *Pseudes* false; Gr. *monas* a unit, monad; M.L. fem. n. *Pseudomonas* false monad', and this etymology was maintained without further comments (Palleroni, 2010).

The genus *Pseudomonas* (*sensu stricto*) is currently restricted to those species related to the type species *P. aeruginosa* within rRNA similarity group I (Palleroni, 1984) or the *fluorescens* rRNA branch (De Vos *et al*., 1985) that belong to the gamma subclass of Proteobacteria (Kesters *et al.,* 1996).

Members of the genus *Pseudomonas sensu stricto* show remarkable metabolic and physiologic versatility, enabling the colonization of diverse terrestrial and aquatic habitats and of great interest because their importance in plant and human diseases, and their growing potential in biotechnological applications (Silby *et al.,* 2011). *Pseudomonas* associated with plants, as other bacterial species, occupy different niches and vary for their effects on plants and coexisting microorganisms. They can act as commensals that have no detectable effect on plant growth or physiology or can act as plant growth-promoting rhizobacteria, by increasing nutrient availability or producing plant growth-enhancing products, and/or as biocontrol agents. They can control plant diseases both indirectly by competing for space and nutrients or stimulating plant defense resistance response and directly by producing antibiotic substances.

Taxonomic definition and revision and species reorganization was largely resolved by DNA-DNA hybridization, RNA analysis, housekeeping gene and multilocus sequencing and recently genome sequences have provided insights into pathogenesis and genetic basis for diversity and adaptation (Silby *et al*., 2011).

Pseudomonas intra-generic phylogenetic relationships analysed on 16S rRNA gene redistributed some of the former *Pseudomonas* species into the *alpha*, *beta* or *gamma* subclasses of *Proteobacteria* and in other genera, e.g. *Burkolderia, Ralstonia, Comamonas* (Kersters *et al.*, 1996; Moore *et al.*, 1996; Anzai *et al.*, 2000)

Phytopathogenic *Pseudomonas* are diverse in the symptoms they induce as well as in their invasion strategies, mechanisms of pathogenesis. The nomenclature and taxonomy of the genus *Pseudomonas* changed over the time. The first comprehensive taxonomic study conducted by Stanier *et al.* (1966) was based essentially of phenotypical and biochemical capabilities. Misaghi and Grogan (1969) analysed nutritional and biochemical comparison of plant-pathogenic and saprophytic pseudomonads and observed that plant pathogenic *Pseudomonas* have a more limited nutritional profile than the saprophytic one that showed high metabolic versatility.

The results of Misaghi and Grogan (1969) adapted to the current *Pseudomonas* species definition restricted most of the *Pseudomonas* plant pathogenic species to the oxidase negative *Pseudomonas* and most of the oxidase positive to plant saprophytic *Pseudomonas*. If we look at studies on plant-associated *Pseudomonas* we become aware that amongst species characterized by the absence of citocrome oxidase (oxidase negative) the most studied and economically important as a plant pathogen with more than 50 pathovars is *Pseudomonas syringae* (Hofte and De Vos, 2006). Oxidase-positive plant pathogenic *Pseudomonas* species include *P. asplenii*, *P. cichorii*, *P. corrugata*, *P. fuscovaginae*, *P. marginalis* pathovars, *P. mediterranea*, *P. palleroniana*, *P. salomonii* and the species pathogenic to cultivated mushrooms, namely *P. agarici*, *P. tolaasii* and *P. constantinii* (Hofte and De Vos, 2006). The most studied saprophytic species such as *P. fluorescens* and *P. putida* have considerable potential for biotechnological applications, particularly in the areas of bioremediation (Kuiper *et al.*, 2004) and as biocontrol agents in plant protection (Mark *et al.*, 2006).

Recently a multilocus sequence analysis (MLSA) approach based on the phylogenetic analysis of the concatenated sequence of 16S rRNA genes, *gyrB*, *rpoD* and *rpoB* of the type strains of 107 *Pseudomonas* species genereated trees which allowed the discrimination of two lineages or intrageneric groups (IG), called IG *P. aeruginosa* and IG *P. fluorescens* (Mulet *et al.*, 2010). The first IG *P. aeruginosa*, was divided into three main groups, represented by the species *P. aeruginosa*, *P. stutzeri* and *P. oleovorans.* The second IG was divided into six groups, represented by the species *P. fluorescens*, *P. syringae*, *P. lutea*, *P. putida*, *P.*

anguilliseptica and *P. straminea*. The *P. fluorescens* group was the most complex and included nine subgroups, represented by the species *P. fluorescens*, *P. gessardi*, *P. fragi*, *P. mandelii*, *P. jesseni*, *P. koreensis*, *P. corrugata*, *P. chlororaphis* and *P. asplenii*. *Pseudomonas rhizospherae* was affiliated with the *P. fluorescens* IG in the phylogenetic analysis but was independent of any group. Some other species were located on phylogenetic branches that were distant from defined clusters (Mulet *et al.*, 2010) (Fig. 1). Plant associated species fall in the *P. fluorescens* lineage and mostly in the *P. fluorescens* and *P. syringae* groups.

The availability of complete genomes represents a revolution in bacterial phylogenetic and evolutionary studies and, consequently, in bacterial taxonomy. The first *Pseudomonas* genome to be completed was that of *P. aeruginosa* PAO1 (Stover *et al.*, 2000) and currently in the *Pseudomonas* genome database (http://www.pseudomonas.com/; Winsor *et al.*, 2009) 44 complete genome of 14 species are listed whereas a total of 538 items are available for complete and draft sequences.

New genome sequence publication is often accompanied with a phylogenetic tree built on the basis of conserved genes in a MLST analysis or on whole proteins comparison that properly allocate the strain in a taxonomic context. Duan *et al.*, (2013) as an example extended the MLST tree of Mulet *et al.* (2010) to 128 strains including also the new available sequences.

Comparing the genomes of a range of *Pseudomonas* encompassing different habitats and behaviors or within the same species has the ambitious goal to define the 'core' and 'accessory' genome of a species but actually any new genome sequence reduce the set of genes in the core genome complement (Silby *et al.*, 2011). Nevertheless is clear that mobile genetic elements such as phages, plasmids, transposons and genomic islands can play a significant role in process such as pathogenesis and antibiotic resistance (Silby *et al.*, 2011). Horizontal gene transfer has impacted the capability of pathogenic *Pseudomonas* spp. in terms of virulence and specificity and genome rearrangement likely contributed to the genetic basis to adaptation and species/strain diversity (Silby *et al.*, 2011).

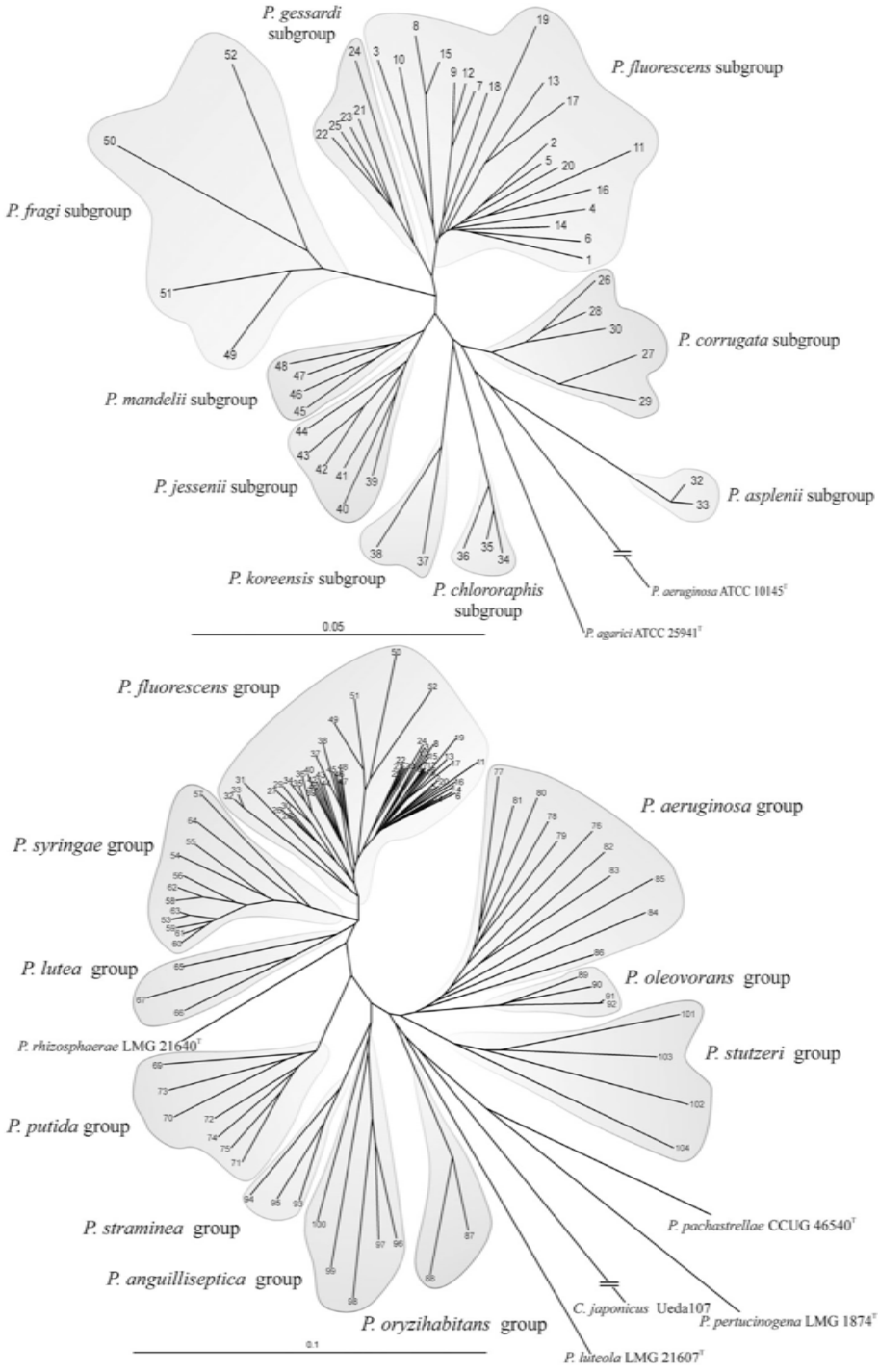


Fig. 1 - Phylogenetic tree (unrooted) of 107 Pseudomonas type strains (A) and of *Pseudomonas fluorescens* group (B) based on the phylogenetic analysis of partial sequences of the 16S rRNA*, gyrB, rpoB* and *rpoD* genes. The bar indicates sequence divergence. Distance matrix was calculated by the Jukes-Cantor method. Dendrogram was generated by neighbour-joining. (Mulet *et al.*, 2010).

1.1.2 Secondary metabolites from biocontrol *Pseudomonas*

The genus *Pseudomonas* comprises multiple species and strains that suppress plant pathogens, promote plant growth, induce systemic resistance, in plants. These strains defined as biocontrol agents produce several diffusible and/or volatile secondary metabolites with antibiotic properties which inhibit pathogens *in vitro* (Haas and Keel 2003; Raaijmakers and Mazzola 2012).

Extensive interest has been devotedto the role of various CLP antibiotics (Raaijmakers *et al*., 2006), siderophores (Cornellis and Matthijs, 2007), hydrogen cyanide (HCN) (Voisard *et al*., 1989), 2,4-diacetylphloroglucinol (DAPG) (Raaijmakers *et al*., 1997), pyrrolnitrin (Howell and Stipanovic, 1979), pyoluteorin (Howell and Stipanovic, 1980), phenazines (Thomashow and Weller, 1988), in biological control of plant pathogens (Fig. 2).

Thomashow and Weller in 1988, for the first time experimentally demonstrated that a *Pseudomonas* antibiotic can suppress plant diseases. They identified in a **phenazinic** compound (phenazine-1-carboxylic acid, PCA), the biocontrol factor produced by *Pseudomonas fluorescens* 2-79. This strain, originally isolated from the rhizosphere of wheat, was found to suppress take-all disease caused by the fungal pathogen *Gaeumannomyces graminis* var. *tritici* (Ggt) on wheat (Haas and Keel, 2003). Phenazines are a large family of antifungal compounds produced by rhizosphere isolates of *P. fluorescens* and *P. chlororaphis*. These group of colorful nitrogen-containing tricyclic molecules, possess antibiotic, antitumor, and antiparasitic activity (Laursen and Nielsen, 2004; Mavrodi and Tomashow, 2006). The unusually broad spectrum activity of phenazines is due to interactions with polynucleotides; topoisomerase inhibition; and the free radicals production which damage several macromolecules and probably might contribute to iron (Fe^{2+}) mobilization in soils from insoluble $Fe(OH)_3$ at neutral pH (Haas and Dèfago, 2005). Phenazines, also inhibit electron transport and are known to have various effects on animal cells, including intercellular signaling involved in transcriptional regulation of the producing cell and having broad effects on bacterial physiology and fitness, including biofilm formation (Maddula *et al*., 2006; Dietrich *et al*., 2008). The core phenazine biosynthetic locus (*phzABCDF*) is highly conserved among phenazine-producing strains of *Pseudomonas* spp., but a range of diverse functions are mediated by auxiliary genes that complement the set of core genes. This auxiliary genes are conserved among all phenazine-producing strains, indicating that they are essential for synthesis of the phenazine scaffold. Regulatory genes belonging to the

luxR/luxI family are also involved in controlling phenazine production in a density-dependent manner, and this system serves as a model for the role of quorum sensing in the ecology of bacteria in natural environments. Analysis of the fully sequenced genomes available to date indicates that the phenazine locus has a complex evolutionary history that includes Horizontal Gene Transfer (HGT) (Gross and Loper, 2009).

Voisard *et al*. (1989) recognized in the **HCN,** a volatile antibiotic compound produced by *P. fluorescens* CHA0, the biocontrol factor that helps to suppress the black root rot disease of tobacco caused by the fungus *Thielaviopsis basicola*. Although HCN is extremely poisonous for its capacity to inhibit the cytochrome c oxidase and other metalloproteins, its production can be beneficial to the host plant because it contributes to the suppression of various plant diseases (Gross and Loper, 2009). HCN in cyanogenic *Pseudomonas* is obtained stoichiometrically from glycine, with production of CO_2 in an oxidative reaction catalyzed by HCN synthase (Laville *et al*. 1998). HCN synthase in *Pseudomonas* is encoded by 3 biosynthetic genes, *hcnA*, *hcnB*, and *hcnC*, organized in an operon structure, which together encode a membrane-bound HCN synthase complex (Laville *et al*., 1998; Pessi and Haas 2000). Among the cyanogenic strains of *Pseudomonas*, the *hcnABC* operon shows highly conserved sequence and organization features, although the genomic context of the operon differs among species (Gross and Loper, 2009).

Pyrrolnitrin is a chlorinated phenylpyrrole compound that use L-Tryptophan as precursor. Its production by *Pseudomonas pyrocinia* was first reported in 1964 by Arima and associates. It shows antagonistic activity against basidiomycetes, deuteromycetes, and ascomycetes, including the plant pathogens *Rhizoctonia solani*, *Botrytis cinerea*, *Verticillium dahliae*, and *Sclerotinia sclerotiorum* (Raaijmakers and Mazzola 2012). Pyrrolnitrin is an inhibitor of fungal respiratory chains (Tripathi and Gottlieb, 1969) and due to its strong antifungal activity, it has been used as an antimycotic in topical preparations for human disease, as well as, synthetic analogues have been developed for use as agricultural fungicides (Gross and Loper 2009). Four biosynthetic genes (*prnABCD*) highly conserved among *P. fluorescens* strains are involved in pyrrolnitrin production. Flanking genes not strictly required for pyrrolnitrin biosynthesis are present, but show regulatory, transport, and biosynthetic functions. To date, *P. fluorescens* Pf-5, 30-84 and O6 are the only pyrrolnitrin-producing Pseudomonad whose complete genomic sequence is available (Loper *et al*., 2012).

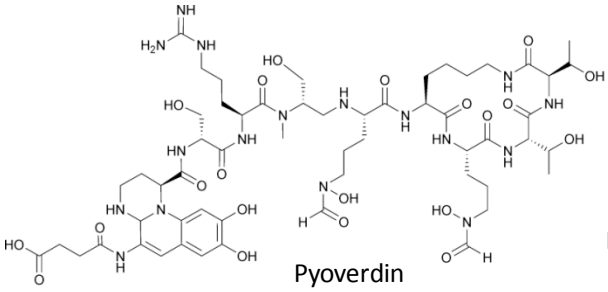
Similarly, **2,4-Diacetylphloroglucinol (DAPG)** is a polyketide phenolic compound, produced by a subset of strains of *P. fluorescens,* toxic toward a wide range of plant

pathogenic fungi and bacteria; possess antihelmintic and, in high concentrations, phytotoxic properties. DAPG is the main factor contributing to biological control of plant disease, inducing systemic resistance and is the primary determinant of the disease suppressive properties of certain soils against the take-all pathogen of wheat (Rezzonico *et al.*, 2007). The DAPG biosynthetic locus has been identified and characterized in several *P. fluorescens* strains, revealing that it is highly conserved among DAPG producers, and comprises not only genes involved in biosynthesis (*phlACBD*), but also efflux (*phlE*), degradation (*phlG*) and regulation (*phlF, phlH*) accessory genes. Phylogenetical analysis revealed that the cluster is ancestral in *P. fluorescens*, and defined by flanking genes, in the genomes of different strains of *P. fluorescens*. Along with the known *phl* biosynthetic genes, located adjacent to the cluster, an additional gene present only in the DAPG-producing strains with unknown functions, was found (Moynihan *et al.*, 2009; Gross and Loper, 2009; Raaijmakers and Mazzola 2012;).

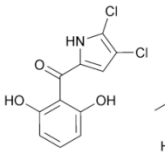
No information is available about the mode of action of **pyoluteorin**. It was first isolated from strains of *P. aeruginosa* and later from many other strains of Pseudomonads (Takeda, 1958; Howell, and Stipanovic, 1980). Pyoluteorin is a hybrid NRPS/PKS natural compound which possesses activity against Oomycetes and in particular against *Pythium ultimum*, an important plant pathogen causing broad-scale economic losses to agriculture. It is also toxic against certain bacteria and fungi and, at high concentrations, exhibits phytotoxicity against certain plants (Maurhofer *et al.*, 1992). The pyoluteorin biosynthetic gene cluster (*plt*), consist of 17 genes spanning a total of about 30 kb and apart from the structural genes (*pltABCDEFGLM*), genes functioning in efflux of pyoluteorin (*pltIJKNOP*) and regulation (*pltZ*, *pltR*) are present in the locus (Haas and Dèfago, 2005; Gross and Loper, 2009).

The majority of fluorescent pseudomonads produce complex fluorescent peptidic **siderophores.** The main ones of the fluorescent *Pseudomonas* spp. are the pyoverdines or pseudobactins which are very efficient iron scavengers (Cornelis and Matthijs 2002). In addition, other siderophores with lower affinity for iron are also produced by pseudomonads, especially by pyoverdin-negative strains. These "secondary" siderophores, include pyochelin, pseudomonine, quinolobactin/thio-quinolobactin, PDTC, corrugatin, ornicorrugatin, yersiniabactin, (Cox *et al.* 1981; Risse *et al.*, 1998; Budzikiewicz 2004; Matthijs *et al.* 2004; Matthijs *et al.* 2008) and possess interesting properties in addition to iron scavenging, such as formation of complexes with other metals or antimicrobial activity (Cornelis and Matthijs

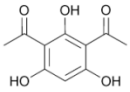
2007). Siderophores, are involved in primary metabolism of bacteria influencing iron acquisition, essential for microbes life, but they also behave as antibiotics and in this case are considered secondary metabolites (Haas and Dèfago, 2005). The ability of bacteria to produce multiple siderophores benefits these organisms, making them more competitive against other organisms in the same niche, and in particular, it is hypothesized that plant growth-promoting rizhobacteria exert their activity by depriving pathogens of iron (Lamont *et al.*, 2002). All fluorescent *Pseudomonas* spp. except for *P. sturzeri*, *P. corrugata* and *P. mediterranea*, secrete an extracellular, yellow-green fluorescent, diffusible pigment siderophore termed **pyoverdine** (Catara *et al.*, 2002; Cornelis and Matthijs, 2002; Gross andl Loper, 2009). This pigment possesses high affinity for Fe^{3+} enabling acquisition of this ion from the environment. It also act as intracellular signaling compound controlling gene expression. Pyoverdines are composed of a chromophore attached to a variable peptide chain both synthesized by NRPSs (Visca *et al.*, 2002; Ravel and Cornelis, 2003). The pyoverdine gene clusters are a component of the core genome common to all fluorescent species. Gene nucleotide composition, of certain genes for pyoverdine biosynthesis and uptake are generally conserved, although the organization of biosynthesic genes in the genomes of different *Pseudomonas* spp. differ significantly. This extraordinary level of variation is in line with the chemical diversity of the pyoverdine structures themselves (Ravel and Cornelis, 2003). In iron poor media *in vitro*, siderophores *Pseudomonas* spp. inhibit the growth of bacteria and fungi and under certain conditions, pyoverdine functions as a diffusible bacteriostatic or fungistatic antibiotic. Another pseudomonad siderophore which possess antifungal antibiotic activity is **pyochelin.** Pyochelin shows a moderate but significantly affinity for Fe^{3+}, whereas strongly chelates divalent metals such as Zn^{2+} and Cu^{2+} forming 2:1 complex, thus it might be able to deprive some fungi of copper and/or zinc (Cox *et al.*, 1981; Schelgel *et al.*, 2004; Tseng *et al* 2006). The pyochelin biosynthetic cluster *phc* consist of two divergent operons *pchDCBA* and *pchEFGHI*, separated by the regulatory gene *pchR*. (Gross and Loper, 2009).



Pyoverdin

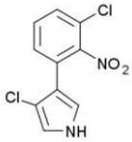


Pyoluteorin

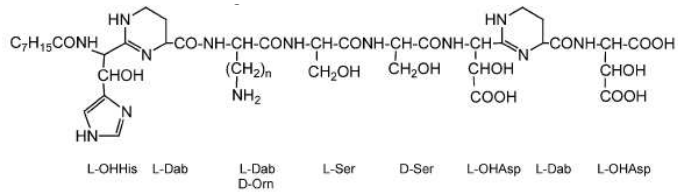


2,4-DAPG

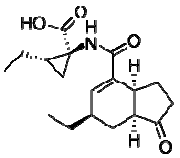
Hydrogen Cyanide



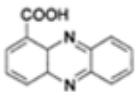
Pyrrolnitrin



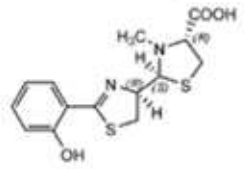
Corrugatin (1, *n* = 2, Dab) and ornicorrugatin (2, *n* = 3, Orn)



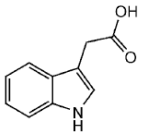
Coronatin
e



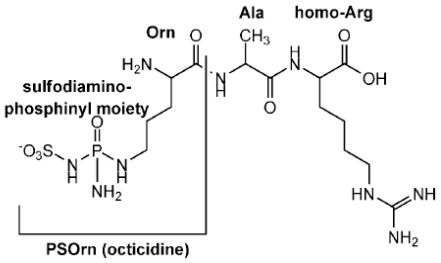
Phenazine-1-carboxylic
acid (PCA)



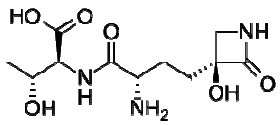
Pyochelin



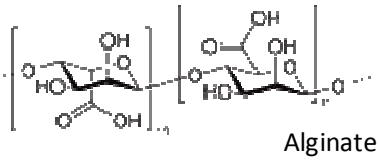
Indole acetic acid
(IAA)



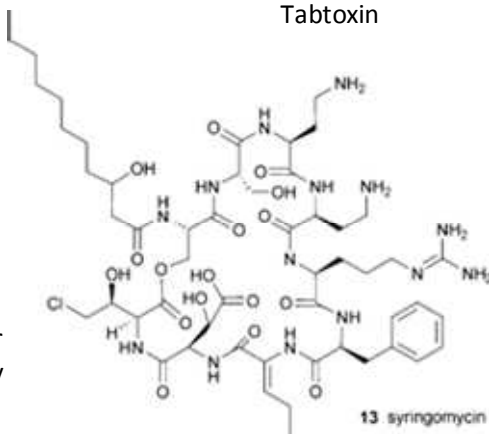
Phaseolotoxin



Tabtoxin



Alginate



Syringomycin

Fig. 2 - Secondary metabolites relevant for biocontrol and for pathogenicity produced by biocontrol and phytopatogenic *Pseduomonas,* respectively.

1.1.3. Toxin production from phytopathogenic *Pseudomonas*

Microbial toxins have been the objects of extensive studies as possible pathogenicity or virulence factors. Bacterial and fungal plant pathogens are known to produce diffusible toxins in infected plants. These phytotoxins are harmful to plants at very low concentrations, and many reproduce at least some of the symptoms of the relevant bacterial or fungal disease. Several phytotoxins have been shown to be involved in pathogenesis. The role of a toxin as a disease determinant is proved by the occurrence of the toxin in an infected plant and the ability of the toxin alone to elicit at least part of the symptoms of the disease, however the interaction between plant pathogens and their hosts is extremely complex. (Graniti, 1991; Slavov, 2005). Phytotoxins act directly injuring plant cells and influencing the course of disease development or symptoms. They also may have host-specificity showing the same specificity of the related pathogen, or exhibit a wider host range of activity.

Pseudomonas produce a wide range of phytotoxic compounds. The most well studied and characterized phytotoxins are those produced by *P. syringae* (Bender *et al.*, 1999). Pathovars of *P. syringae* produce a variety of secondary metabolites toxins, in infected plants and in culture media, with diverse chemical structures and active at very low concentrations. *P. syringae* phytotoxins, although not essential for pathogenicity (some disease can occur in their absence), generally act as virulence factors that cause or increase disease severity (such as chlorosis or necrosis) in infected plants. *P. syringae* phytotoxins are also involved in systemic movement and multiplication of pathogen *in planta* and in lesion size (Bender *et al.*, 1999; Hofte and de Vos, 2006) (Fig. 2).

Antimetabolite toxins are the major group of phytotoxins produced by different *P. syringae* pathovars. These oligopeptides (tabtoxin, phaseolotoxin and mangotoxin) inhibit enzymes involved in the biosynthetic pathways of some amino acids (Fig 3).

Tabtoxin is phytotoxic dipeptide from *P. syringae* with a β-lactam structure, consisting of tabtoxine- β-lactam (TβL) linked to a threonine (Levi and Durbin, 1986). The phytotoxin is secreted by *P. syringae* pv *tabaci*, pv. *coronafaciens* and pv. *garcae*, and associated with the symptoms of wildfire on tobacco and halo blight of oats and coffee, respectivelty (Durbin *et al.*, 1978). The toxin is activated only after hydrolysis of the peptide bond with aminopeptidases present in either bacteria or the plant, yelding the TβL toxic moiety. TβL irreversibly inhibit the glutamine synthase, resulting in the reduced availability of the enzyme which is the only way to efficiently detoxify ammonia (Uchytil and Durbin, 1980).

Tabtoxin biosynthesis proceeds along the lysine pathway (*dabABCDE*) and the biosynthetic cluster has been reported to excise readily from the chromosome of *P. syringae* pv. *tabaci*. The locus exhibits a patchy distribution among the major groups of *P. syringae* (Gross and Loper,2009).

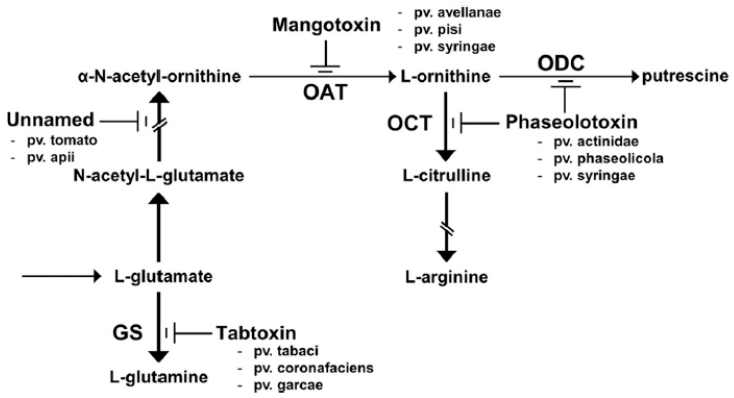


Fig. 3 - Schematic representation of the arginine-glutamine and polyamine biosynthesis pathways. The enzymatic targets that are inhibited by antimetabolite toxins mangotoxin, phaseolotoxin, tabtoxin, and an unnamed phytotoxin that was produced by different *P. syringae pathovars are shown. Target enzymes have* the following abbreviations: GS, glutamine synthetase; OAT, ornithine N-acetyltransferase; OCT, ornithine carbamoyltransferase; and ODC, ornithine decarboxylase. The pathovars of *P. syringae* producing each toxin are indicated. (Carrion *et al.*, 2013).

Phaseolotoxin is produced by *P. syringae* pvs. *phaseolicola* and *actinidiae*, causal agents of halo blight of beans and bacterial canker of kiwifruit respectively. Mitchell (1976) and Moore *et al.* (1989) elucidated the structure of phaseolotoxin, which consist of a sulfodiaminophosphinyl moiety linked to a tripeptide (ornithine, alanine, and homoarginine). Phaseolotoxin is a protoxin, that competitively inhibit the ornithine carbamoyl transferase (OCTase). Plants peptidases convert the toxin in octicidine or PSorn (N^{δ}-(*N*sulfodiaminophosphinyl)-L-ornithine) which is a potent irreversible inhibitor of OCTase. Inhibition of OCTase determines the accumulation of ornithine and of arginine, leading to chlorosis symptoms (Mitchell and Bieleski, 1979; Ferguson and Johnston, 1980). A nonribosomal thiotemplate (NRPS) mechanism was propesed for the assembly of the tripeptidic backbone of phaseolotoxin, but the corresponding NRPS genes as well as the biosynthetic precursor of the sulfodiaminophosphinyl moiety, have not yet been identified (Gross and Loper, 2009).

P. syringae pv. *syringae* strains isolated from mango, as well as those isolated from other plants hosts such as tomato, produce the antimetabolite toxin **mangotoxin.** It inhibits ornithine acetyl transferase, a key enzyme in the biosynthetic pathway of ornithine and arginine (Arrebola *et al*. 2003; Cazorla *et al*. 2003). Mangotoxin structure remains unknown; however, preliminary chromatographical and biochemical characterization showed that it is very similar to the antimetabolite tabtoxin and phaseolotoxin. Arrebola *et al*. (2007) demonstrated the involvement of a NRPS in mangotoxin production and, reported that mangotoxin-defective mutants, displayed a clear delay in the initiation and development of the necrotic symptoms on tomato leaves, indicating in mangotoxin a virulence factor for *P. syringae* (Arrebola *et al*., 2011; 2009). Additionally, mangotoxin, like other antimetabolite toxins produced by *P. syringae* pathovars, shows antimicrobial activity against some bacteria that usually colonize the aerial parts of plants suggesting that its production is involved in the epiphytic fitness of this bacterium in the phyllosphere. Recently, draft genomes investigations together with the *mbo* operon phylogenetic analysis, showed that mangotoxin is produced by five *P. syringae* pathovars: *aptata*, *avellanae*, *japonica*, *pisi* and *syringae*; and that this ability has probably been horizontally acquired only once during the evolution of this bacterial species (Carrión *et al*., 2013).

Coronatine is a chlorosis-inducing non host-specific phytotoxin produced by several pathovars of *P. syringae* (*artropurprurea*, *glycinea*, *maculicola*, *morsprunorum* and *tomato*) (Mitchell, 1982; Mitchell *et al*., 1983; Wiebe *et al*., 1993). It functions as a virulence factor, stimulating the stomata opening facilitates the entrance of the pathogen in the host plant and suppress the salycilic acid-mediated host defense (Melotto *et al*., 2006; Brooks *et al*., 2005). Coronatine shows a close structural and functional homology to plant horomones ethylene and jasmonic acid, suggesting that it impact the ethylene and jasmonate signaling pathway involved in plant defense against herbivoides and certain pathogens (Uppalapati *et al*., 2005). The hybrid NRPS-PKS mechanism of synthesis has been suggested for coronatine, in fact the compound presents two distinct components: the polyketide coronofacic acid and the coronamic acid. Biosynthesis proceeds by formation of these two moieties, which are then linked to produce coronatine. In many strains of *P. syringae* the *cor* biosynthetic gene cluster is in the flexible genome, carried on large plasmids (pPT23A family) that are transferred *via* conjugation (Bender *et al*., 1999; Gross and Loper 2009).

Toxins such as syringomycins and syringopeptins, have been extensively discussed in the literature. These toxins are responsible for necrotic symptoms by inducing pore

formation and disrupting plant cell membrane functions. They belong to the **cyclic lipopeptides (CLPs**), class of toxins. CLPs, are secondary metabolites with different roles, often unique to the biology of the producing microorganism. As consequence of their structural diversity, CLPs are surface active molecules with antibacterial, antifungal, citotoxic and phytopathogenic properties, in fact are considered major virulence factors in several *Pseudomonas* species, including *P. corrugata*, *P. fuscovaginae*, *P. tolaasii* and *P. fluorescens* (*marginalis*). CLP characteristics will be discussed in more detail below.

Other secondary metabolites that have a role in plant-bacteria interaction include hormones, siderophores, polysaccharides, enzymes, glycoprotein.

Among the plant hormones produced by pathovars of *P. syringae* and related species the **Indole-3-acetic acid (IAA)** is the most studied and its involvement in *P. savastanoi* pv. *savastanoi* pathogenicity has been unambiguously demonstrated. IAA (auxin class of hormones) is considered a virulence factor leading, together with cytochines, to the formation of the characteristics knots on olive and oleander (Surico *et al.*, 1985). In bacteria, the biosynthesis of IAA proceeds from L-tryptophan to IAA *via* the IAM pathway, which was first identified in *P. savastanoi* and comprise two genes organized in one operon, *iaaH* and *iaaM.* The operon is commonly accompanied by *iaaL* which is involved in IAA pool size regulation and virulence. In strains of *P. savastanoi*, the *iaaL–iaaHM* cluster can be located on the chromosome or on conjugative plasmids, which are widely distributed in phytopathogenic *Pseudomonas* spp. and are known to carry genes required for virulence and ecological fitness (Metal *et al.*, 2009; Gross and Loper 2009). There are also indications that IAA may inhibit plant defense mechanisms (Robinette and Matthysse, 1990).

Wilting induced by vascular pathogens and water soaking associated with foliar pathogens is due to **exopolysaccharide (EPS)** polymers production by phytopathogenic bacteria. Levan and alginate are the two main EPS molecules usually produced by *P. syringae* pathovars (Gross and Rudolph, 1987). However, alginate appears to be the major EPS produced in water soaked lesions (Fett and Dunn, 1989; Rudolph *et al.*, 1989). *P. syringae* alginate biosynthetic gene cluster was cloned and characterized (Peñaloza-Vàzquez *et al.*, 1997) and it showed high homology with that one of *P. aeruginosa*. However, the regulation and signals involved in alginate biosynthesis differed in the two species, probably as consequence of their adaptation to plant and animal hosts, respectively (Peñaloza-Vàzquez *et al.*, 1997). Alginate plays a role in epiphytic fitness and contributes to the virulence of *P. syringae* pv. *syringae*, perhaps by facilitating colonization or dissemination of the bacterium

in planta (Yu *et al.*, 1999). *P. syringae* pv. *ciccaronei*, which is the pathogenic agent responsible for the leaf spots of carob plants, produce the phytotoxic mannan exopolysaccharide, which induced chlorosis and necrosis on tobacco leaves (Corsaro *et al.*, 2001).

Soft-rotting *Pseudomonas fluorescens* (*marginalis*) strains are capable of degrading pectic components of plant cell walls by producing a wide variety of **pectolytic enzymes** (pectin methyl esterase, pectin lyase, polygalacturonase and two pectate lyase isozymes). *P. viridiflava* produces a single pectate lyase (PelV), and at least some of the *P. syringae* pathovars also produce pectic enzymes. *pel* gene sequences are available in the database for *P. syringae* pv. *lachrymans*, *P. syringae* pv. *phaseolicola*, *P. syringae* pv. *tabaci*, and *P. syringae* pv. *glycinea*. The *P. viridiflava pelV* gene has been mutated revealing it to be essential for soft-rot pathogenesis (Hoft and de Vos, 2006).

P. syringae and a few other bacterial species including strains of *P. fluorescens* and *P. viridiflava* possess the ability to nucleate supercooled water to form ice (Lindow *et al.*, 1982). However strains within pv. *syringae* frequently exhibit the **ice nucleation ability (INA**), while none of the strains tested thus far within pv. *tomato* or *morsprunorum* show the ice phenotype (Hoft and de Vos, 2006). The presence of INA bacteria on leaf surfaces can destroy leaf habitats and are responsible for ice formation, and hence injury to plants, mainly in the range from 0 to –5°C (Hirano and Upper, 2000). Ice nucleation activity is quantitatively quite variable in *P. syringae* strains. It has been demonstrated that *P. syringae* pv. *syringae* B728a possesses an unlinked gene encoding an antifreeze protein (Feil *et al.*, 2005) able to modulate the formation of external ice.

1.2. The role of cyclic lipopetides (CLP) in plant-microbe interactions

Among the bacterial Lipopeptide (LPs) producers, *Pseudomonas* and *Bacillus* have received the most attention. Both genera are predominant in various natural habitats harbouring pathogenic and beneficial species, and exhibiting diverse lifestyles. (Bender *et al.*, 1999; Nybroe & Sørensen, 2004; Raaijmakers *et al.*, 2006; Ongena & Jacques, 2008; Gross & Loper, 2009).

These secondary metabolites possess amphipathic structure containing both a hydrophilic peptide portion and a hydrophobic fatty acid portion (Roongsawang *et al.*, 2010).

LP production was first reported from Gram-positive *Bacillus subtilis* IAM1213 and after which, various types of LPs with significant surface and/or antimicrobial properties have been isolated and characterized from several *Bacillus* strains. *Pseudomonas* mainly produce lipopeptides with a cyclic structure (CLPs) characterized by the presence of a lacton ring in the peptide chain and have recently, been classified, into six main groups and as 'other' (Gross and Loper; 2009; Roongsawang *et al.*, 2010).

CLPs are generally synthesized in a ribosome-independent manner by megaenzymes called nonribosomal peptide synthetases (NRPSs) (Marahiel *et al.*, 1997). Gene clusters encoding NRPSs for CLP production have been cloned and characterized, demonstrating that transcriptional induction, post-translational modification and assemblage are necessary to obtain an active-form product, but also their versatility and evolutionary lineages (Roongsawang *et al.*, 2010).

Throughout this thesis I use CLPs in a broader sense; LPs will be used only for characteristic molecules.

1.2.1 Structure

A variety of methods have been used to elucidate the primary and secondary structures of CLPs produced by bacteria, including Nuclear Magnetic Resonance analysis (NMR), Mass Spectromettry techiniques (MALDI-TOF/MS, HPLC), and chemical methods (Ballio *et al.*, 1996; Bare *et al.*, 1999; De Souza *et al.*, 2003; Henriksen *et al.*, 2000; Koumoutsi *et al.*, 2004; Monti *et al.*, 2001; Nielsen *et al.*, 1999, 2002; Sørensen *et al.*, 2001). CLPs produced by *Pseudomonas* spp. are composed of a fatty acid tail linked to a short oligopeptide, which is cyclized to form a lactone ring between two amino acids in the peptide chain. Based on the length and composition of the fatty acid tail as well as the number, type, and configuration of the amino acids in the peptide moiety, CLPs of *Pseudomonas* spp. have been classified into six groups :viscosin, syringomycin, amphisin, putisolvin, tolaasin, and syringopeptin groups (Gross and Loper; 2009) (Fig. 4).

The viscosin and amphisin group consist of CLPs with 9 and 11 amino acids (AA) respectively in the peptide moiety linked at the N-terminus to, in most cases, 3-hydroxy decanoic acid (3-HDA). *Pseudomonas* spp. producing CLPs in the **viscosin** group are from a variety of origins, including soil, rhizosphere, phyllosphere, and marine environments. For

example, viscosin production has been described for pectolytic strains of *P. fluorescens* causing head rot of broccoli (Hildebrand *et al.*, 1998) and for a *Pseudomonas* isolate obtained from a tube worm collected from a marine environment (Gerard *et al.*, 1997). Similarly the CLP surfactant massetolide A also belonging to viscosin group was first identified in cultures of a marine *Pseudomonas* sp. isolated from the surface of a leafy red alga (Gerard *et al.*, 1997); and subsequently identified in other two biocontrol strains; *P. fluorescens* SS101 (De Souza *et al.*, 2003), and strain MF-30 (Konnova *et al.*, 2004). In the viscosin group is also included another CLP structurally similar to viscosin called white line–inducing principle (WLIP), but they differ from each other for the chirality of leucine in position 5 which has D configuration in WLIP and L configuration in viscosin (Coraiola *et al.*, 2006). WLIP, is produced by the so called "*Pseudomonas reactans*" strains. When *P. reactans* is confronted with *Pseudomonas tolaasii* on solid medium, a white line precipitate is produced between the colonies, as consequence of the interaction of WLIP with tolaasin produced by *P. tolaasii* (Wong and Preece 1979). Recently the NRPS system for biosynthesis of WLIP in a *P. putida* strain (RW10S2) has been identified and confirmed that it is required for the white-line reaction (Rokni *et al.*, 2012).

For several members of **amphisin** group, including amphisin and tensin, the crystal structure has been resolved (Henriksen *et al.*, 2000; Sørensen *et al.*, 2001). Nielsen and associates (2002) identified several other interesting characteristics for the *P. fluorescens* strains producing amphisin-like CLPs, including the production of chitinases and the volatile hydrogen cyanide (HCN). These two traits have been associated with biocontrol of plant-pathogenic fungi and, in particular, HCN production is not common among the *P. fluorescens* strains producing viscosin-like CLPs (Raaijmakers *et al.*, 2006). In recent years, a number of structurally related CLPs have been identified and characterized for *Pseudomonas*, including arthrofactin from *Pseudomonas* (formerly Arthrobacter) sp. strain MIS38 (Morikawa *et al.*, 1993; Roongsawang *et al.*, 2003).

Tolaasin group harbors CLP with heterogeneous structure due to variations in both the composition and length of the peptide chain (18 to 22 AA) and the lipid tail (3-HDA or 3-hydroxyoctanoic acid [3-HOA]). The peptide part of the CLPs in this group contains several unusual amino acids, such as 2,3- dihydro-2-aminobutyric acid (Dhb) and homoserine (Hse), the first always being in front of the *allo*-Thr residue. The cyclic part of the peptide moiety contains five amino acids and the lactone ring is formed between the C-terminal amino acid and the *allo*-Thr residue (Raaijmakers *et al.*, 2006).

Group/Name	Structure
Viscosin[A]	
Viscosin	FA β OH L Leu D Glu D *a*Thr D Val L Leu D Ser L Leu D Ser L Ile
Viscosinamide	FA-β-OH-L-Leu-D-Gln-D-*a*Thr-D-Val-L-Leu-D-Ser-L-Leu-D-Ser-L-Ile
Massetolide A	FA-β-OH-L-Leu-D-Glu-D-*a*Thr-D-*a*Ile-L-Leu-D-Ser-L-Leu-D-Ser-L-Ile
Pseudophomin A	FA-β-OH-L-Leu-D-Glu-D-*a*Thr-D- Ile-D-Leu-D-Ser-L-Leu-D-Ser-L-Ile
Pseudodesmin A	FA-β-OH-L-Leu-D-Gln-D-*a*Thr-D-Val-D-Leu-D-Ser-L-Leu-D-Ser-L-Ile
Syringomycin[B]	
Syringomycin A	FA-β-OH-L-Ser-D-Ser-D-Dab-L-Dab-L-Arg-L-Phe-Z-Dhb-L-Asp$_{(3\text{-}OH)}$-L-Thr$_{(4\text{-}Cl)}$
Syringostatin A	FA-β-OH-L-Ser-D-Dab-L-Dab-D-Hse-L-Orn-L-*a*Thr-Z-Dhb-L-Asp$_{(3\text{-}OH)}$-L-Thr$_{(4\text{-}Cl)}$
Syringotoxin B	FA-β-OH-L-Ser-D-Dab-L-Gly-D-Hse-L-Orn-L-*a*Thr-Z-Dhb-L-Asp$_{(3\text{-}OH)}$-L-Thr$_{(4\text{-}Cl)}$
Pseudomycin A	FA-β-OH-L-Ser-D-Dab-L-Asp-D-Lys-L-Dab-L-*a*Thr-Z-Dhb-L-Asp$_{(3\text{-}OH)}$-L-Thr$_{(4\text{-}Cl)}$
Cormycin A	FA-β-OH-L-Ser-D-Orn-L-Asn-D-Hse-L-His-L-*a*Thr-Z-Dhb-L-Asp$_{(3\text{-}OH)}$-L-Thr$_{(4\text{-}Cl)}$
Amphisin[A]	
Arthrofactin	FA-β-OH-D-Leu-D-Asp-D-*a*Thr-D-Leu-D-Leu-D-Ser-L-Leu-D-Ser-L-Ile-L-Ile-L-Asp
Amphisin	FA-β-OH-D-Leu-D-Asp-D-*a*Thr-D-Leu-D-Leu-D-Ser-L-Leu-D-Gln-L-Leu-L-Ile-L-Asp
Lokisin	FA-β-OH-D-Leu-D-Asp-D-*a*Thr-D-Leu-D-Leu-D-Ser-L-Leu-D-Ser-L-Leu-L-Ile-L-Asp
Pholipeptin	FA-β-OH-D-Leu-L-Asp-L- Thr-D-Leu-D-Leu-D-Ser-D-Leu-D-Ser-D-Leu-L-Ile-D-Asp
Tensin	FA-β-OH-D-Leu-D-Asp-D-*a*Thr-D-Leu-D-Leu-D-Ser-L-Leu-D-Gln-L-Leu-L-Ile-L-Glu
Putisolvin[C]	
Putisolvin I	$CH_3(CH)_4CO$-D-Leu-D-Glu-D-Leu-D-Ile-D-Gln-D-Ser-D-Val-D-Ile-D-Ser-L-Leu-L-Val-X-Ser
Putisolvin II	$CH_3(CH)_4CO$-D-Leu-D-Glu-D-Leu-D-Ile-D-Gln-D-Ser-D-Val-D-Ile-D-Ser-L-Leu-L-Xle-X-Ser
Tolaasin[A]	
Tolaasin I	FA-β-OH-Dhb-Pro-Ser-Leu-Val-Ser-Leu-Val-Val-Gln-Leu - - - - Val-Dhb-*a*Thr-Ile-Hse-Dab-Lys
Fuscopeptin	FA-β-OH-Dhb-Pro-Leu-Ala-Ala-Ala-Ala-Val-Gly-Ala-Val-Ala - - - Val-Dhb-*a*Thr-Ala-Dab-Dab-Phe
Corpeptin	FA-β-OH-Dhb-Pro-Ala-Ala-Ala-Val-Val-Dhb-Hse-Val-aIle-Dhb-Ala-Ala-Ala-Val-Dhb-*a*Thr-Ala-Dab-Ser-Ile
Syringopeptin[A]	
SP22	FA-β-OH-Dhb-Pro-Val-Val-Ala-Ala-Val - - - Val-Dhb-Ala-Val-Ala-Ala-Dhb-*a*Thr-Ser-Ala-Dhb-Ala-Dab-Dab-Tyr
SP25	FA-β-OH-Dhb-Pro-Val-Ala-Ala-Val-Leu-Ala-Ala-Dhb-Val-Dhb-Ala-Val-Ala-Ala-Dhb-*a*Thr-Ser-Ala-Val-Ala-Dab-Dab-Tyr
SP25[Phe$_{25}$]	FA-β-OH-Dhb-Pro-Val-Ala-Ala-Val-Leu-Ala-Ala-Dhb-Val-Dhb-Ala-Val-Ala-Ala-Dhb-*a*Thr-Ser-Ala-Val-Ala-Dab-Dab-Phe
Other	
Entolysin[D]	FA-β-OH-D-Xle-D-Glu-D-Gln-D-Val-D-Xle-D-Gln-D-Val-D-Xle-D-Gln-D-Ser-L-Val-L-Xle-D-Ser-X-Xle
Ofamide[A]	FA-β-OH-L-Leu-D-Glu-D-*a*Thr-D-*a*Ile-L-Leu-D-Ser-L-Leu-L-Leu-D-Ser-L-Val
Pseudofactin[A]	$CH_3(CH)_{14}CO$-X-Gly-X-Ser-X-Thr-X-Leu-X-Leu-X-Ser-X-Leu-X-Leu/Val
Syringafactin[E]	FA-β-OH-D-Leu-D-Leu-D-Gln-L-Leu-D-Thr-L-Val-D-Leu-L-Leu

[A] The hydroxyl group of *a*Thr or Thr form an ester bond with the carboxyl group of the *C*-terminal amino acid
[B] The hydroxyl group of L-Ser$_3$ form an ester bond with the carboxyl group of the *C*-terminal amino acid
[C] The hydroxyl group of D-Ser$_6$ form an ester bond with the carboxyl group of the *C*-terminal amino acid
[D] The hydroxyl group of D-Ser$_{10}$ form an ester bond with the carboxyl group of the *C*-terminal amino acid
[E] Linear lipopeptide

Fig. 4 - Primary structures of representative cyclic lipopeptides (CLPs) produced by *Pseudomonas* spp. (Roongsawang *et al.*, 2010).

Several tolaasin like CLPs are produced by plant-pathogenic strains of *Pseudomonas* and constitute important virulence factors. Tolaasin group comprises molecules such as fuscopeptins (19 AA) produced in culture by the gramineae pathogen *P. fuscovaginae* (Ballio *et al.*, 1996), corpeptins (22 AA) produced in culture by *P. corrugata,* causal agent of tomato pith necrosis (Emanuele *et al.*, 1998) and tolaasins (18 AA) produced in culture by *P.tolaasii* , the causal agent of brown blotch disease of *Agaricus bisporus* and of the yellowing of *Pleurotus ostreatus* (Lazzaroni *et al.*,2003).

CLP siringopeptins were included by Raaijmakers and associates (2006) into the tolaasin group. Recently Gross and Loper (2009) reclassified CLPs including **siringopeptins** in

a distint group. They, in fact, contain a larger peptide moiety, with 22 or 25 AA, linked to the same lipid tail of CLP of toolasin group (i.e., 3-HAD, 3-HOA), but form a lactone ring of 8 AA (Gross and Loper, 2009; Lazzaroni *et al.*, 2003). The phytotoxic and necrosis inducing CLP syringopeptins are produced in culture by *P.syringae* pv. *syringae* and are the longest CLPs described.

CLPs belonging to **putisolvin** group (putisolvins I and II) consist of a C_6 lipid moiety and a 12-AA peptide (Kuiper *et al.*, 2004; Raaijmakers *et al.*, 2006; Gross and Loper, 2009). Cyclization occurs between the C-terminal carboxyl group and the ninth amino acid residue, instead of the first or third amino acid in other *Pseudomonas* CLPs. The putisolvins I and II were the first known CLPs to have a peptide chain of 12 AA and the two structures differ only at the second amino acid from the C-terminus, which is Val for putisolvin I and Leu/Ile for putisolvin II (Kuiper *et al.*, 2004; Raaijmakers *et al.*, 2006; Gross and Loper, 2009). Putisolvins are produced by *Pseudomonas putida* strain PCL1445 isolated from roots of plants, grown on a site polluted with polycyclic aromatic hydrocarbons (Kuiper *et al.*, 2004).

Syringomycin-like CLPs are nonapeptides (9AA) which include in their structure unusual AA like the 2,4 – diamino buytic acid (Dab) and at the C-terminal a chlorinated threonine residue, that is for antifungal activity of syringomycin (Grgurina *et al.*, 1994) and for cyclization reaction. The fatty acid tail of CLPs in the syringomycin group may consist of a 3-hydroxy or 3,4-dihydroxy fatty acid composed of 10 to 14 carbon atoms. Syringomycin is representative of the cyclic nonapeptide class, but characteristically, three forms of syringomycin are produced, differing only by the length of the 3-hydroxy fatty acid moiety: SR-A1, SR-E, SR-G (Bender *et al.*,1999). CLP cormycin A (CM-A) from *P. corrugata* which shares several structural features with other nonapeptides from *P. syringae* spp. (Scaloni *et al.*, 2004) is also included in this CLP group.

In addition to these six main CLP groups, other linear and cyclic lipopeptides identified in *Pseudomonas* spp., but not sharing structural similarity with CLPs included in the main groups, have been grouped in a class defined as "**other**". This group reports molecules with no cyclic strucuture like syringafactin or structurally unrelated CLPs like entolysin and orfamide.

1.2.2. Biosynthesis

For both genera *Bacillus* and *Pseudomonas* most CLPs are synthesized by large nonribosomal peptide synthetases (NRPSs) via a thiotemplate process. NRPSs are not limited by the restrictions of the "ribosome's rulebooks" and are also involved in the production of several other unusual and potent peptides, including drugs such as penicillin, cyclosporin and vancomycin (Marahiel *et al.*, 1997; Raaijmakers *et al.*, 2006).

These multienzyme complexes possess a modular structure, with each module catalyzing the addition and modification of a specific amino acid. Their substrates are not restricted to the usual proteinogenic amino acids but also can incorporate D-amino acids, carboxy acids, or fatty acids (Gewolb 2002; Sieber and Marahiel 2005). The number and the sequence of AA in the peptide moiety of the final CLP molecule usually consist with the number and order of the modules in the NRPS, a phenomenon known as the ''colinearity rule'' (Fig. 5). A typical NRPS module consists of a minimal set of three domains. An adenylation domain (A) of about 550 AA residues highly conserved and repeated which shares significant homology with the family of acyl-CoA synthases and luciferases. It is responsible for the AA selection and activation as an amino acyl adenylate. Because signature sequences within the A domain are specific to a given amino acid, the amino acid composition of the peptide product can be predicted bioinformatically. This activated AA is directly transferred downstream of the A domain, to the thiolation domain (T), also called peptidyl carrier protein (PCP). The T domain is the site of 4'-PP cofactor and is an integral part of these multienzymes with a functional unit of about 100 AA residues, to which aminoacyl substrates are bound as thioesters. A condensation (C) domain (about 450 AA) catalyzes peptide bond formation between the amino acid present on the T of the same module and the peptidyl intermediate bound to the T domain of the preceding module to elongate the peptide chain (Marahiel *et al*, 1997; Donandio *et al*, 2007; Gross and Loper 2009). Usually, all elongation modules present these core domains. These catalytic domains generate a linear peptide which is cleaved at the end of the assembly line by a thioesterase (TE) domain, which results in the release of a linear product or a cyclic compound via an intramolecular cyclization reaction (Cy domain) (Sieber and Marahiel 2005). Additional domains may be present, such as epimerization (E) responsible for conversion of regular L-configured amino acids into the corresponding D-form; methylation (M) and reductase (R)

domains involved respectively in N-methylation and reduction activities (Donadio *et al.*, 2007).

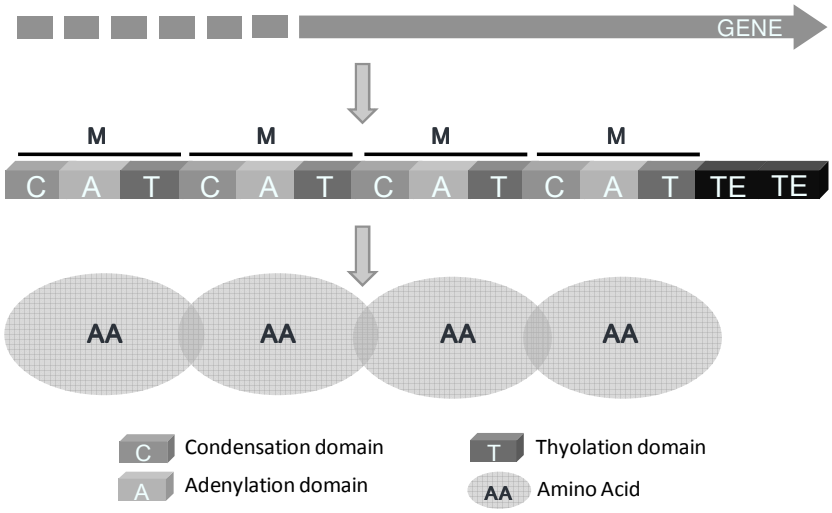


Fig. 5 - Schematic diagram showing the module and domain organization of a non ribosomal peptide synthetase (NRPS). Usually the order of the modules in the biosynthetic gene cluster indicates the order of the amino acids in the polypeptide. This phenomenon is called 'colinearity rule'. The amino acid-activating modules are arranged in the order that corresponds to the amino acid sequence of the peptide.

With a DNA region encompassing 74 kb and carrying 22 NRPS modules, the *P. syringae* pv. *syringae* B301D syringopeptin (*sypABC*) biosynthesic cluster represents the largest DNA regions dedicated to encoding a peptide synthetase in prokaryotes. Analysis of the predicted SypA, SypB, and SypC proteins indicated that all three proteins contain multiple amino acid activation modules typical for peptide synthetases, accounting for all 22 amino acids found in the syringopeptin structure (Scholz-Schroeder *et al.*, 2003).

In contrast to *Bacillus*, no E domains have been found so far in CLP biosynthetic templates of *Pseudomonas*, species. Roongsawang *et al.* (2003) initially postulated that external racemases may be responsible for the conversion of AA from L – to D-configuration in arthrofactin produced by *Pseudomonas* reflecting the recognition of sequence differences downstream of a conserved core motif in the T domains. For massetolides of *P. fluorescens* strain SS101, however, no relationship could be established between this sequence motif and the amino acid configuration (De Bruijn *et al.*, 2008). Subsequent studies on arthrofactin

biosynthesis indicated that in *Pseudomonas* the D-configuration of the AA in the CLPs can be generated by C domains that have dual catalytic activities (i.e. condensation and epimerization), referred to as C/E domains involved in epimerization of the amino acid that is loaded onto the T domain of the preceding module (Balibar *et al.*, 2005). This assumption was consistent for most of the individual C domains of the massetolide, arthrofactin, syringomycin and syringopeptin biosynthesis genes (Balibar *et al.*, 2005; De Bruijn *et al.*, 2008). However the predicted dual catalytic activity did not match the configuration of the amino acid for two C domains (C2, C6) of the massetolide biosynthesis cluster (De Bruijn *et al.*, 2008); and other exceptions were found by Balibar *et al.* (2005) for syringopeptin suggesting that these C/E domains could also function as dual condensation/dehydration domains with or without prior epimerization.

Analyses of the metabolite profiles of *Pseudomonas* and *Bacillus* species show that single strains can simultaneously produce representatives of various CLP families, but also multiple structural analogues of one particular CLP. For example, *B. subtilis* can produce 12 surfactin analogs differing in the nature of the peptide residues and/or in the length and branching of the fatty acid chain (Kowall *et al.*, 1998). Also *P. fluorescens* strain SS101 produces at least eight structural analogs of massetolide A (Gerard *et al.*, 1997). The observations that CLP-biosynthesis mutants do not produce the main CLP or any of the CLP derivatives indicate that these analogues are the result of the flexibility in amino acid selection and activation by the A domains. Substrate flexibility of A domains is a common phenomenon in nonribosomal peptide synthesis and, instead of being considered a 'mistake' of the A domains, this may have biological functions for the producing strain (Raaijmakers *et al.*, 2010).

1.2.3 *Pseudomonas* CLP biosynthetic clusters

Analysis of gene clusters and genome sequences of various *Pseudomonas* species and strains known to produce CLPs revealed that genes encoding functionally connected enzymes often form clusters or operon structures. The genetic organization of CLP biosynthetic loci reveals that several genes flanking the synthetic genes are conserved among *Pseudomonas* CLP biosynthesis clusters, including the two genes encoding the putative CLP transporter that are usually located downstream of last gene of synthetase and

regulatory genes, and LuxR-type transcriptional regulators positioned up- and downstream of the CLP biosynthesis genes involved in CLP production regulation (de Bruijn, *et al.,* 2009)(Fig. 6). Similar genes have been identified in arthrofactin (Roongsawang, *et al.,* 2003), syringopeptin and syringomycin (Scholz-Schroeder, *et al.*, 2001; Feil *et al.*, 2005), massetolide A (de Bruijn, *et al.*, 2008), and putisolvin (Dubern, *et al.*, 2008) biosynthesis gene clusters.

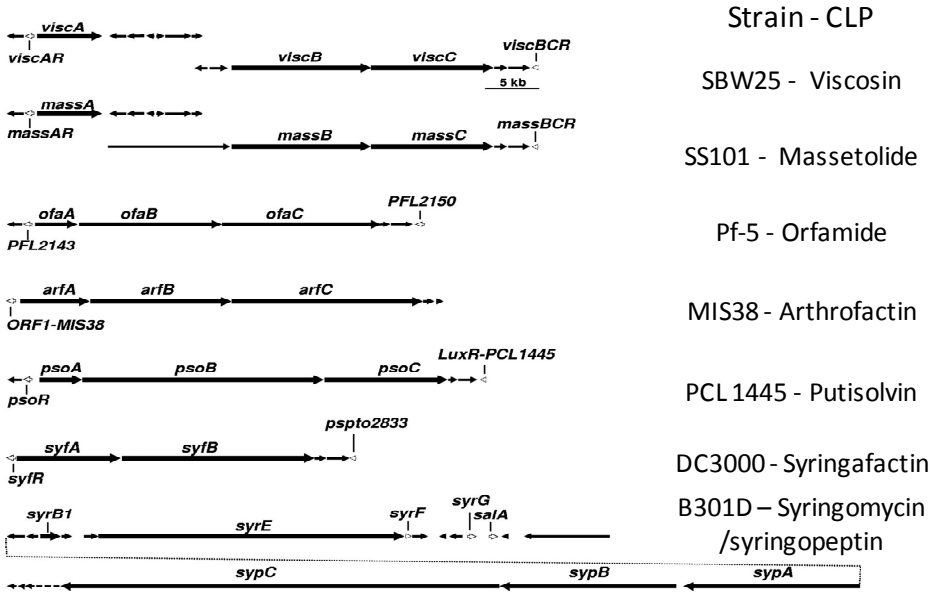


Fig. 6 - Schematic presentation of various CLP biosynthesis genes and their flanking genes in *Pseudomonas* species and strains (De Bruijn and Raaijmakers, 2009a).

1.2.3.1. Syringomycin and syringopeptin

Syringomycin and syringopeptin are two class of CLP phytotoxins and key determinant of *Pseudomonas syringae* B301D virulence (Bender *et al.*, 1999).

Syringomycin peptide moiety consist of nine amino acids (AA) and is synthesized by two NRPSs (SyrB1, SyrE) and three modifying protein systems (SyrB2, SyrC, SyrP). SyrB1 and SyrE do not follow the co-linearity rule and also lack E-domains. SyrE harbors eight modules arranged in a line for the first eight AA, but the ninth module encoded by *syrB1* and necessary for incorporation of the last amino acid (L-Thr9) is located in the upstream region. Accessory proteins SyrB2 is non-heme Fe(II) halogenase and is involved in chloronitration of L-Thr9 activated and loaded by SyrB1. This intermediate is transferred from the T-domain of SyrB1 to SyrE by aminoacyltransferase SyrC to form the final product. SyrP whose gene is

located upstream of *syrB1* catalyze hydroxylation of Asp at module 8. Although three AA residues in the peptide are in the D-form, no E-domains are associated with the modules incorporating the respective D-AA. Balibar and associated (2005) in fact demonstrated that SyrE contains unique dual C/E domains, which contribute to the conversion of L-AA to the D-form.

Syringopeptin consist of a large peptide moiety with 22 or 25 AA residues (Scholz-Schroeder *et al.*, 2003). The biosynthesis is governed by three NRPS: SypA, SypB, SypC and the order and the number of modules follow the co-linearity rule. Although several D-AA are present in the sequence no E-domains are contained in Syp synthetase. In contrast to the Syr synthetase, SypC contains two unique *C*-terminal Te-domains predicted to catalyze the release and cyclization of syringopeptin.

1.2.3.2. Arthrofactin

Arthrofactin is a cyclic lipoundecapeptide (11AA) produced by *Pseudomonas* sp. MIS38, and its biosynthesis is catalyzed by a synthetase consisting of three NRPS ArfA (243kDa), ArfB (474kDa) and ArfC (648kDa) containing two, four and five modules respectively (Roongsawang *et al.,* 2003, 2011). Arf contains no E- domains, as found in syringomycin and syringopeptin synthetase, despite the presence of seven AA residues in arthrofactin are in the D-form. Balibar and associates demonstrated in fact that Arf contains unique dual C/E domains involved in the conversion of L-AA in D-AA. This novel C/E domain is cryptically embedded with the C-domain located downstream of the D-amino acid–incorporating modules and can be recognized by an elongated His motif (HHI/LXXXXGD). This feature is also present in the Syr and Syp synthetases. Arf also presents *C*-terminal tandem Te-domains like syringopeptin. As demonstrtated by site-directed mutagenesis only the first Te-domain (ArfC-Te1) is essential for the cyclization and release of the final product, whereas the second one (ArfC-Te2) seems to be involved in the evolution of Arf to improve the macrocyclization efficiency.

1.2.3.3. Viscosin and Massetolide

Viscosin (*P. fluorescens* SBW25) and massetolide (*P. fluorescens* SS101) are structurally related lipononapeptides (9AA) synthesized by NRPS systems, encoded by *viscA*/*viscB*/*viscC* and *massA*/*massB*/*massC* respectively (de Bruijn, *et al.,* 2007; 2008). The

viscA/massA gene is not clustered with the other two genes (*viscBC* and *massBC*) but is located at a different locus of the *Pseudomonas* genome. AA sequences analysis revealed two modules in ViscA/MassA, four modules in ViscB/MassB, and three modules in ViscC/MassC. Tandem Te-domains were identified in the last ViscC/MassC module and are likely to be functional for the biosynthesis of both lipopeptides as was shown for the two Te-domains in arthrofactin synthetase. Dual C/E domains similar to that one of arthrofactin were found.

1.2.3.4. Orfamide

The orphan gene cluster involved in orfamide biosynthesis includes three large ORFs termed *ofaA/B/C* discovered from the *P. fluorescens* Pf-5 genome using a''genomisotopic approach,'' in which genome sequence analysis togetgher with a isotope-guided fractionation is employed to identify unknown compounds synthesized from orphan gene clusters containing nonribosomal peptide synthetases (Gross *et al.*, 2007). Orfamide is a cyclic lipodecapeptide and although is composed of 10 amino acids, its structure is similar to lipononapeptide viscosin, suggesting a common linear evolutionary lineage. Orfamide biosynthetic cluster OfaA/B/C consist of ten modules and no cognate E-domains are found in Ofa modules. Ofa seems to contains six dual C/E domains although only five amino acid residues are in D-form, suggesting that the NRPS system is quite complex. Tandem TE-domain are present in the C-terminus of OfaC, similar to NRPSs from other *Pseudomonas*.

1.2.3.5. Putisolvin

P. putida PCL1445 produce two isoforms of putisolvin (I and II) a cyclic lipododecapeptide (Kuiper *et al.,* 2004). The biosynthetic cluster consists of three genes *psoA/B/C* codifying for two, seven and three functional modules respectively. Nine of the 12 amino acids are in D-form in fact dual C/E domains are organized downstream of the first nine modules. Sequence analysis also revealed that the first nine T-domains in Pso synthetase are responsible for transferring D-aminoacids. Prediction of A-domain substrate specificity in the eleventh module indicates its preference for Val over Leu or Ile, which correlates well with the production ratios of putisolvin I and II. PsoC carries putative tandem TE-domains (Dubern *et al.,* 2008).

1.2.3.6. Syringafactin

Syringafactin is a novel linear lipopeptide produced by *P. syringae* pv. *tomato* DC3000 consisting of an eight-amino acid linear peptide linked to a β-hydroxy fatty acid. Genome mining of *P. syringae* pv. *tomato* DC3000 identified the biosynthetic cluster (Berti *et al.,* 2007). Three and five NRPS modules are encoded by two genes respectively *syfA* and *syfB.* SyfA presents the The *N*-acyl domain indicating that syringafactin would contain an *N*-terminal fatty acid chain; SyfB contains tandem Te-domains at the *C*-terminus. Based on the location of the dual C/E domains, the The D/L-configuration of each residue has been tentatively assigned, giving this hypothetical structure to the syringafactin fatty acyl-D-Leu1-D-Leu2-D-Gln3-Leu4-D-Thr5-Val6-D-Leu7-Leu8. Amino acid sequence analysis also revealed highest level of similarity between ArfA from *Pseudomonas* sp. MIS38 and SyfA, suggesting that the syringafactin NRPS system in *P. syringae* pv. *tomato* DC3000 probably evolved from the arthrofactin system, after which three modules of the arthrofactin NRPS were deleted. Deleted modules include residues involved in cyclization of arthrofactin, justifying the linear form of syringafactin.

1.2.3.7. Entolysin

Entolysin is a cyclic lipotetradecapeptide (14AA) with a quite small lacton ring (4AA) produced by an entomopathogenic bacterium *Pseudomonas entomophila*. Entolysin exhibits similar characteristics described for other CLP, but it is not involved in the virulence of the producing strain for killing *Drosophila*. The biosynthetic cluster consists of three genes (*etlA*, *etlB*, and *etlC*). It is high similar to the putisolvin synthetase in the closely related strain *P. putida* PCL1445, but the three genes are not physically linked like reported for the viscosin and massetolide gene clusters. EtlA, EtlB, and EtlC comprise two, eight, and four functional NRPS modules, corresponding to the number of amino acid residues in the product peptide. These modules are composed of typical domains, but no E-domains have been identified, whereas the C12 and C13 could function as dual C/E domains. Tandem Te-domains were also identified in the EtlC *C*-terminus (Vallet-Gely *et al.*, 2010).

1.2.4 Mechanism of action

The toxins that form channels can destabilize the membrane forming mainly two types of pore. The pore "barrel stave" that is an aqueous channel characterized by helices associated to form a bundle with a central lumen, like a barrel made of helical peptides staves; and the “toroidal pore” made by the peptide associated with the lipid head groups even when they are perpendicularly inserted in the lipid bilayer allowing the contact of the inner and outer leaflets of the membrane bilayer (Muller *et al.,* 2000; Yang *et al.*, 2001) (Fig. 7). Biological and artificial membranes (planar lipid bilayer, vescicles and liposome with different lipid composition) red blood cells from differen origins, plant protoplasts, are usually employed in biophysical studies to investigated the CLP pore forming properties. The small size of the CLP, not enough to pass completely through the membrane suggest the possible involvement of lipids in the bilayer process of formation of the pore, promoting the lipid transfer between the inner and the outer membrane layer. This was a clear indication that a mixed lipid/peptide pore with “toroidal” structure is formed (Hutchinson, *et al.*, 1995; Dalla Serra *et al.*, 1999; Menestrina *et. al.*, 2003; Scaloni *et al.*, 2004; Coraiola *et. al,* 2006).

The polar peptide head with the lipophilic fatty acid tail is responsible for the amphiphilic properties of CLPs, which can lower surface tension and interact with cellular membranes, thereby altering their integrity. This latter effect is assumed to contribute to interactions with other organisms and in general to be involeved in plant pathogenicity, antifungal, antibacterial, antiviral and phosphatidylinositol-specific phospholipase (PIPLC) inhibitory activity (Gross and Loper 2009).

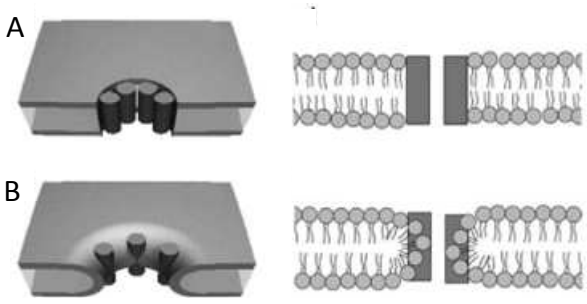


Fig. 7 - Schematic illustration of the transmembrane pores induced in lipid bilayers: a barrel-stave model (A) and a toroidal model (B). Protein monomers are shown as dark cylinders (left) and as dark rectangles (right).

Among the most well-characterized bacterial CLP, syringomicin and syringopeptins produced by the plant pathogen *Pseudomonas syringae*, mechanism of action is probably the well studied and investigated system.

Plasma membrane of host cells is the primary target of syringomycin, in fact the amphipathic structure of the lipopeptide promotes its insertion into the lipid bilayers of membranes causing pores forming and increasing of transmembrane fluxes of K^+, H^+ and Ca^{2+} both lethal to cells. Pore forming activity was demonstrated by Hutchison *et al.* (1995) in a pure black-lipid membrane and afterwards showed that this process is highly efficient and occurs with nanomolar amounts of syringomycin as demonstrated in assays of tobacco protoplasts (Hutchison and Gross, 1997). Takemoto and associates demonstrated that an influx of H^+ appears to be accompanied by an efflux of K^+ across the syringomycin channel. These cations exchange generates an electrochemical gradient with consequent collapse of the pH gradient of the plasma membrane, resulting in acidification of the cytoplasm (Bender *et al.*, 1999). The most conspicuous effect of channel formation is also a rapid and sustained influx of Ca^{2+} ions that activates a cascade of events associated with cellular signaling in plants, such as induction of kinase-mediated phosphorylation of membrane proteins (Bidwai and Takemoto, 1987). The ultimate benefit to the bacterium from pore formation is the systematic release of nutrients into the intercellular spaces of host tissues (Hutchinson *et al.*, 1995) and the alkalization of intercellular fluids, resulting in a more favorable environment for bacterial growth (Che *et al.*, 1992).

Biophysical analysis in planar lipid bilayer membranes clarified how syringomycin pores function and are formed (Kaulin *et al.*, 1998). After insertion into the lipid bilayer, monomers of syringomycin aggregate into pore complexes: lipophilic portion of toxin subunit resides in the core of the bilayer, whereas the hydrophilic peptide head resides close to the surface of the membrane. Individual channels can become aggregated into clusters that exhibit synchronous opening and closing. Based on the voltage-dependent behavior of ion channels, Feigin *et al.* (1996) deduced that at least six molecules of syringomycin are involved in the channel formation. A pivotal influence in channel formation by syringomycin is certainly given by lipid bilayer composition. Julmanop *et al.* (1993) and Taguchi *et al.* (1994) reported that sterols, particularly ergosterol, promote the binding of syringomycin to cells; whereas sphingolipids, which are major lipid components of eukaryotic plasma membranes, have been associated with cell sensitivity to syringomycin (Cliften *et al.*, 1996; Grilley *et al.*, 1998). In fact a *SYR2* mutant of the fungus *Saccharomyces cerevisiae* is highly

resistant to syringomycin due to a failure to produce 4-hydroxylated sphingolipids such as phytoceramide (Grilley *et al.*, 1998).

Similarly to syringomycin the amphiphilic characteristics suggest that syringopeptins function as a membrane-permeabilizing toxins. The phytotoxic activity of syringopeptin is centered on the ability to form pores in plant plasma membranes, thereby promoting transmembrane ion flux and cell death. In fact syringopeptin appears to induce an H^+-K^+ exchange response in plant cells (Mott and Takemoto, 1989), and to cause electrolyte leakage of plant cells (Iacobellis *et al.*, 1992) like syringomycin, although several studies reported syringopeptin to be more active than syringomicin (Iacobellis *et al.*, 1992; Lavermicocca *et al.*, 1997). As a pore forming CLP, syringopeptin is able to form ion channels in black-lipid membranes, to lyse both tobacco protoplasts and erythrocytes, and to generate a rapid and sustained influx of $^{45}Ca^{2+}$ across the plasma membrane of tobacco protoplasts (Hutchinson and Gross, 1997).

Hutchinson and Gross (1997) predicted the biophysical characteristics of syringopeptin pores suggesting that aggregates of metabolite monomers are required for pore formation but fewer molecules of syringopeptins may be necessary to form a functional pore, probably due to the larger charged head than syringomicin. Recently Bensaci and associates (2011) investigated the channel-forming properties of the syringopeptins in lipid bilayers in comparison with those of syringomycin. It was observed that syringopeptins induced voltage-dependent conductance in planar lipid bilayers influenced by sphingolipid and sterol presence in the yeast membrane. A similar mechanism of action was demonstrated for syringomycin suggesting that syringopeptins form stable membrane pores comprised of and modulated by host membrane lipids and particularly sphingolipids and sterol (Malev *et al.*, 2002; Takemoto *et al.*, 2003; Kaulin *et al.*, 2005). Interestingly syringopeptins turned out to be ~150 times more effective than syringomycin in inducing macroscopic conductance but forming single channels with similar properties. It is not known precisely how the syringopeptins and syringomycin interact with membrane lipids to form channels (Malev *et al.*, 2002). However, the electrostatic and hydrophobic interactions between SP22A and membrane lipids are known to increase lipid ordering as a prelude to stable pore formation (Szabo *et al.*, 2004).

Studies on biological properties of cormycin from *P. corrugata* reported new interesting insights into previous information on structure–function relationships regarding these molecules. Two main structural features have been proposed to strongly affect the

biological activity of *Pseudomonas* spp. CLPs, i.e. the occurrence of basic residues and the presence of specific hydrophobic moieties. The positive charges should also facilitate interaction with negatively charged phospholipids, allowing CLP insertion itself into membrane bilayers. However, other important physicochemical phenomena should be considered to clarify the mechanism of action of these molecules. Scaloni *et al.,* (2004) in fact, showed that although peptides belong to the same chemical class possess different biological activity as consequence of the different charge properties.

The activity of the WLIP and tolaasin I, produced by virulent strains of *P. reactans* and *P. tolaasii*, respectively, was comparatively evaluated on lipid membranes. Both CLPs were able to induce the release of calcein from large unilamellar vesicles, although in most of the cases tolaasin I displayed higher porating activity. Their activity was dependent on the toxin concentration and liposome composition and in particular it increased with the sphingomyelin content of the membrane (Coraiola *et al.,* 2006). Fuscopeptins (FPs) are produced by the plant pathogen *Pseudomonas fuscovaginae*. As expected from the structural relationship between FPs and the other peptin-like CLPs, they display hemolytic activity on human erythrocytes and were able to induce calcein release from LUVs, with an effect FPs concentration and lipid composition dependent. FPs are able to open pores on pure POPC (1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine) membranes In line with those reported for tolaasin by Coraiola and associated (2006) where a prevalence of helical structure was indeed observed in lipid membrane, the authors speculate that lipid molecules could participate in the formation of the lesion and cooperate with the peptide to the polar surface of the pore, according to the toroidal pore model already proposed for syringomycin and syringpeptins (Coraiola *et al.*,2008).

At high concentrations (well above the critical micelle concentration [CMC value]), CLPs can directly solubilize plasma membranes. In several studies (Bender *et al.* 1999), solubilization of membranes also was demonstrated for erythrocytes. Interestingly, erythrocyte hemolysis by tolaasin can be inhibited by addition of divalent metal ions such as Zn^{2+}, Ca^{2+}, and Mg^{2+} (Rainey *et al.* 1991). Given that pre-incubation of tolaasin with Zn^{2+} did not affect its ability to lyse erythrocytes and that cells pretreated with Zn^{2+} were no less susceptible to tolaasin induced lysis than untreated cells, Rainey and associates (1991) proposed that the inhibitory effect of divalent metal ions operates by binding to negatively charged groups on the extracellular side of the plasma membrane close to the site of pore formation. Studies with surfactin (Dufour *et al.*, 2005) further indicated that the cyclic

character of the peptide moiety is important for erythrocyte hemolysis because linear products of surfactin failed to cause lysis.

1.2.5 Regulation

Relatively little is known about the genetic network involved in the signal transduction pathways and the perception of external factors (abiotic and biotic) that drive transcription of the CLP biosynthetic genes. A number of recent studies have highlighted that several global regulatory mechanisms and specific transcriptional regulators located in proximity of the CLP biosynthesis genes are involved in the regulation of production in *Pseudomonas* spp. (Raaijmakers *et al.*, 2010).

The GacA/GacS two-component regulatory system seems to work as a master switch, in fact mutants disrupted in either one of the two genes resulted impaired in CLP production. This regulatory system consists of a membrane-bound sensor kinase protein (GacS) and a cytoplasmic response regulator protein (GacA). The two-component GacS/GacA system is critical for the regulation of syringomycin and syringopeptin toxins in *P. syringae* pv. *syringae* B301D and appears to be at the top of the regulatory hierarchy controlling their production, involving also the downstream regulator SalA (Kitten *et al.*, 1998 ; Willis and Kinscherf 2004, Wang *et al.*, 2006). Very little is known about signals that serve as a trigger for CLP biosynthesis. Gross and colleagues showed that specific phenolic β-glycosides (e.g. arbutin) and specific sugars common to plant tissue enhanced expression of the *syrB* gene increasing production of syringomycin. Amphisin synthesis was also demonstrated to be strictly dependent on GacS in *Pseudomonas* sp. DSS73 a strain isolated from the sugar beet rhizosphere and even induction by seed exudate depends on a functional GacS locus (Koch *et al.* 2002). As the active compound(s) in the seed exudates were heat stable and could be removed by dialysis, therefore Koch *et al.* (2002) suggested that small organic molecules may be involved, not excluding a role for inorganic compounds such as micronutrients. Regulation by GacA/GacS two component system was also demonstrated for putisolvins (Dubern *et al.* 2005), massetolide A, and viscosin (de Bruijn *et al.*, 2007, 2008).

In addition to two-component regulation, another well-known regulatory system, referred to as quorum sensing, has also been investigated for a number of CLP–producing species and strains. In many *Pseudomonas* species and in general in Gram negative bacteria,

the most common signal molecules used are N- acylated homoserine lactones (AHLs), which synthase is encoded by *luxI*- type genes. At the critical cell density, when the quorum concentration of AHLs has been reached, AHLs interact directly with the LuxR-type protein; thus the LuxR-AHL complexes can then bind at specific promoter DNA sequences called *lux*-boxes of QS regulated genes affecting their expression (Venturi, 2006).

In the plant pathogenic *P. fluorescens* strain 5064 and saprophytic *P. putida* strain PCL1445, N-AHL-based quorum sensing was shown to be involved in viscosin and putisolvin biosynthesis, respectively (Cui *et al.*, 2005; Dubern *et al.*, 2006). Screening of six mutants of strain 5064 affected in AHL production (N-3-acyl-hydroxyoctanoyl-AHL) showed that they were also impaired in viscosin production, and the addition of the culture extracts or the synthetic signal molecules restored viscosin biosynthesis in the mutants (Cui *et al.*, 2005). Dubern and associates (2006) found in strain PCL1445 four N-AHLs associated with regulation of putisolvin biosynthesis, and mutation in both *ppuI* and *ppuR* (*luxI* and *luxR* homologous) abolishes putisolvin production almost completely. No information was found on the role of the quorum sensing in amphisin (Andersen *et al.*, 2003), viscosin (De Bruijn *et al.*, 2007), massetolide (De Bruijn *et al.*, 2008) biosynthesis.

Particular attention has also been given to the LuxR-type transcriptional regulators positioned up- and downstream of the CLP biosynthesis genes. This kind of LuxR-type regulators contain the typical DNA-binding helix-turn-helix (HTH) motif in the C-terminal region, but do not harbour the autoinducer-binding domain typical of the quorum-sensing-associated LuxR regulators (Wang *et al.*, 2006; De Bruijn and Raaijmakers, 2009a, b). These regulators seem to be involved in the biosynthesis of syringomycin, syringopeptin, syringafactins, putisolvins, viscosin and massetolide (Lu *et al.*, 2002; Wang *et al.*, 2006; Berti *et al.*, 2007; Dubern *et al.*, 2008; De Bruijn & Raaijmakers, 2009a).

Wang *et al.* (2006) showed that SalA and SyrF are critical for the coregulation of syringomycin and syringopeptin production and that together with the sensor kinase GacS mediate transmission of the plant signal molecules to the *syr-syp* biosynthesis cluster. Both SalA and SyrF belong to a family of transcriptional activators characterized by high sequence similarities to the C-terminal region of LuxR, which contains a helix-turn-helix (HTH) domain. The authors demonstrated that, both SalA and SyrF are similar to LuxR proteins with regard to dimerization and interactions with promoter regions of target genes. SalA regulates the *syr-syp* genes by forming a dimer and interacting with the *syrF* promoter. SyrF then activates the *syr-syp* genes directly by binding to their promoter regions.

Two ORFs encoding for LuxR-like transcriptional regulators located upstream of and downstream of non ribosomal peptide biosynthetic gene cluster encoding for proteins involved in the production of six structurally related linear lipopeptides were identified in *Pseudomonas syringae* pv. *tomato* DC3000. Analysis on mutant strains on both regulators revealed that only the regulator located upstream plays a clear role in lipopeptide production (Berti *et al.,* 2007). Dubern and collaborators (2008) identified in *P. putida* PCL1445 a luxR-type regulatory genes *psoR,* located upstream of putisolvin cluster and required for its expression. A mutation in PsoR abolished putisolvin production, giving the first evidence of its regulatory role in putisolvin biosynthesis. The decrease of expression of the *psoA* : : *gfp* fusion in *psoR* mutant when compared to the wild-type suggested that the effect of *psoR* on putisolvin production can be accounted for by its effect on *psoA* transcriptional activity, although PsoR may regulate other genes involved in putisolvin production as well.

Phylogenetic analysis of these LuxR-type proteins showed that LuxR-type regulators located upstream of the CLP biosynthesis genes clustered separately from the LuxR-type regulators located downstream of the CLP biosynthesis genes, except for the LuxR-type regulators SalA, SyrG, and SyrF from *P. syringae* pv. *syringae*, which were dispersed among the two clusters. Other well-known LuxR-type regulators, including AHL binding regulators (i.e. RhlR and LasR from *Pseudomonas aeruginosa*, and LuxR from *V. fischeri*), autonomous effector domain regulator (i.e. GerE from *B. subtilis*) and response regulators activated upon phosporilation (i.e. FixJ from *S. meliloti*) clustered distantly can be classified as a separate subfamily of LuxR-type regulators.

De Bruijn & Raaijmakers, (2009a) established that viscosin production in *P. fluorescens* SBW25 is also regulated by two LuxR-type genes and demonstrated that LuxR-type transcriptional regulators can be exchanged among different *Pseudomonas* strains, thereby regulating the biosynthesis of structurally different CLPs.

A number of other genes were recently identified that regulate CLP production in *Pseudomonas*. DnaK in *P. putida* is a member of the heat shock protein (Hsp70) and is positively regulated by the GacA/GacS system (Dubern *et al*., 2005, Raaijmakers *et al*., 2006, 2010). Mutation of *dnaK* gene in strain PCL 1445 strongly reduced putisolvin I and II production, and further investigations on the role of temperature on metabolite production, revealed that putisolvin production is upregulated at low temperatures and that *dnaK* is required for putisolvin production at low temperatures. Sequencing both up- and downstream of the *dnaK* gene further led to the identification of two other genes, *dnaJ* and

grpE, that both adversely affected the synthesis of putisolvin. Although the exact role of these three genes in regulation of putisolvin production is not yet known, it was postulated that they may be required for the proper folding or activity of other positive regulators of the putisolvin synthetic gene *psoA*, or that DnaK complex interact with the *rpoS* encoded sigma factor σ^S, or is necessary for the correct assembly of the peptide sinthetase complex.

In *P. fluorescens*, random mutagenesis led to the identification of a regulator of massetolide biosynthesis: *clpP* (De Bruijn and Raaijmakers, 2009b). ClpP is a serine protease that is highly conserved in bacteria and eukaryotes, and it is also important for virulence in several bacterial pathogens. Together with other proteases, ClpP plays a pivotal role in intracellular refolding and degradation of proteins. ClpP protease regulates the biosynthesis of cyclic lipopeptide surfactants that play an important role in swarming the motility, biofilm formation, and antimicrobial activity of *P. fluorescens* SS101. At the transcriptional level, ClpP-mediated regulation of massetolide biosynthesis appeared to operate independently by the Gac system. Based on these findings, a tentative model was proposed where ClpP regulates, alone or together with a chaperone other than ClpX, massetolide biosynthesis via degradation of putative transcriptional repressors of *massAR* or via modulation of the citric acid cycle and amino acid metabolism.

1.2.6 Activity

The biological activity of CLPs and other biosurfactants seems to be related to their physical and chemical properties and in recent years, studies on mutants deficient in CLP production for several *Pseudomonas* strains allowed to highlight on the natural roles of these secondary metabolites. Four main potential fuctions are associated to CLPs produced by *Pseudomonas* spp.:

- ✓ Role in plant interaction: pathogenesis and induction of systemic resistance
- ✓ Antimicrobial activity
- ✓ Regulation of attachment and detachment to and from surfaces
- ✓ Motility

1.2.6.1. Role in plant interaction: pathogenesis and induction of systemic resistance

CLPs produced by plant-pathogenic *Pseudomonas* species are important, although not the solely required metabolites in the infection process (Bender *et al.*, 1999).

The contribution of syringopeptin and syringomycin to the virulence of *P. syringae* pv. *syringae* strain B301D was examined in immature sweet cherry with syringopeptin *(sypA)* and syringomycin *(syrB1)* synthetase mutants defective in the production of the two toxins. *sypA* and *syrB1* mutants were reduced in virulence 59% and 26%, respectively, compared with the parental strain in cherry, whereas the syringopeptin–syringomycin double mutant was reduced 76% in virulence (Scholz-Schroeder *et al.*, 2001a). Little is known about role of fuscopeptins of *P. fuscovaginae*. A comparative study between some biological activities of purified syringomycin-E (SR), syringopeptin 22-A (SP) and fuscopeptin A (FP) demonstated that it is involved in induction of necrotic lesions on rice leaf sheath and on tobacco leaves (Ballio *et al.*, 1996). Toxicity assays of the two substances WLIP (*Pseudomonas reactans*) in comparison with tolaasin I (*P.tolaasii*), either on whole sporophores on tissue blocks and whole sporophores of *Agaricus bisporus* showed that the antifungal activity of both toxins, which caused significant brown and sunken lesions on the surface of the above *Agaricus* tissue blocks and on whole sporophores, although at different concentrations (Lo Cantore *et al.,* 2006).

Similarly for plant-pathogenic *P. fluorescens* 5064 viscosin does not appear to play a direct role in virulence in fact a viscosin-deficient mutant was still able to cause decay of the broccoli tissue, although confined to the wounded florets (Hildebrand *et al.*, 1998). In addition toxic effects of CLPs on plant cells, their surfactant properties play an important indirect role in virulence by facilitating colonization of plant tissue and enhancing physical access of cell-wall-degrading enzymes (CWDE) to the plant surface (Hildebrand *et al.* 1998; Hutchison and Johnstone 1993; Lindow and Brandl 2003). Pore formation, the alkalization of the intercellular fluid and the release of intracellular compounds, represent favorable conditions for multiplication and increased CLP production by the invading bacteria. These events seems to be pivotal for pathogenesis role of CLPs (Raaijmakers *et al.*, 2006).

Recently it has also shown that CLPs have beneficial effects through the stimulation of the plant immune system by inducing defence responses in plants against pathogenic fungi and oomycetes. For example purified massetolide A of *P. fluorescens* on leaves determined enhanced resistance to infection by *P. infestans* (Tran *et al.*, 2007).

1.2.6.2. Antimicrobial activity

CLPs exhibit lytic and growth inhibitory activity against a wide range of microorganisms, i.e. viruses, mycoplasmas, bacteria, fungi and oomycetes (Raaijmakers *et al.*, 2006). A number of studies confirmed the importance of structural characteristics for their antagonistic activity, such as the fatty acid chain length and the charge of the peptide moiety. The antiviral activity of CLP was first reported by Groupé and colleagues (1951) (reviewed in Nybroe and Sørensen, 2004), in which viscosin was shown to have activity against human-pathogenic enveloped viruses, including the infectious bronchitis virus.

Syringomycin and syringopeptins from *P. syringae*, exhibit antimicrobial activity against a number of filamentous fungi, bacteria and yeasts. It has been demonstrated the *in vitro* fungicidal efficacy of syringomycin against fungi, including, *Geotrichum candidum, Penicillum digitatum, Aspergillus* species and yeasts, such as *Saccharomyces cerevisiae* and *Rhodotorula pilimanae* (Zhang *et al.*, 1987; Lavermicocca *et al.*, 1997; Bull *et al.,* 1998, Bender *et al.,* 1999). Interesting results have also been obtained *in vivo* toward the clinically important fungi, *Candida* spp. (Sorensen *et al.*, 1998). The related nonapeptides STs and SSs have nearly equivalent broad-spectrum antifungal activity. Syringopeptins show an antimicrobial spectrum of activity distinct from that of syringomycin and related cyclic lipononapeptides. Some strains of *Botrytis cinerea* are highly sensitive to syringopeptins, whereas *G. candidum* is resistant. *Bacillus megaterium* was the most sensitive to syringopeptins and so, this bacterium is used in routine bioassays of syringopeptin activity (Bender *et al.*,1999; Lavermicocca *et al.*, 1997). Cirvilleri *et al.*, (2005) evaluated the *in vitro* inhibition activity of a total of 71 *P. syringae* pv. *syringae* strains obtained from herbaceous and woody plant species. All the strains inhibited, to a different extent, all the tested microorganisms: *R. pilimanae*, *B. megaterium*, *Salmonella* sp., *Listeria monocytogenes*, *P. syringae*, *Botrytis cinerea, Alternaria alternata, Aspergillus ochraceus, Fusarium solani*. Lops (2005) also demonstrated the influence of the media in the *in vitro* inhibitory behavior of screened 33 strains of nine pathovars of *P. syringae* and in any case IMMA medium (minimal medium) appeared the best substrate for inducing CLP production. Fuscopeptin (*P. fuscovaginae*), showed similar biological properties to other long chain CLPs and in particular to be active toward *B. cinerea*, moderately active and inactive toward *R. pilimanae* and *G. candidum* respectively (Ballio *et al.*, 1996).

Tolaasin I (*P. tolaasii*) inhibited the growth of a wide range of microorganisms including filamentous fungi, gram-positive bacteria and some gram-negative bacteria belonging to genera *Erwinia*, *Escherichia*, *Agrobacterium*, *Pseudomonas*, and *Xanthomonas* (Lo Cantore *et al*., 2006). Of particular interest is the activity towards some soil-inhabiting phytopathogenic fungi and chromista and the cultivated mushrooms *Agaricus bisporus*, *Pleurotus* spp., and *Lentinus edodes*. Furthermore, tolaasin I inhibited the growth of *C. albicans* and *C. neoformans*, but the sensitivity of the above fungi was lower when compared to that of phytopathogenic fungi. WLIP (*P. reactans*) inhibited the growth of *Bacillus megaterium* ITM100, and further assays showed that crystalline WLIP possess activity against fungi, chromista, and gram-positive bacteria. No activity against most of the tested gram-negative bacteria was detected, with the only exception for a strain of *Erwinia carotovora* subsp. *carotovora* (Lo Cantore *et al*., 2006). Massetolide and viscosin, showed activity against *Mycobacterium tubercolosis*, *Mycobacterium avium-intercellular* and purified viscosinamide inhibited the *in vitro* and *in vivo* growth of *Rizhoctonia solanii* (Gerard *et al*., 1997; Nielsen *et al*., 1999).

The apparent lack of activity of several CLPs agaist gram negative bacteria has been ascribed to protective effect of the outer membrane (Nybroe and Sørensen 2004). Putisolvins produced by *P. putida* PCL1445 do not show activity against *P. fluorescens* and *P. aeruginosa* (Kuiper *et al*.,2004); whereas toolasin possess activity against several Gram negative bacteria (Lo Cantore *et al.,* 2006).

Recently the studies on the antifungal activity of CLPs focus not only on the spectrum of activity and the minimal concentration for inhibiting fungal growth, but also on their effects on fungal morphology and physiology (Raaijmakers *et al*., 2010).

CLPs from *Pseudomonas* have also significant impact on Oomycetes such as *Pythium* and *Phytophtora* species. The impact on zoospores lysis has been well characterized for viscosin, viscosinamide, massetolide A, putisolvins and orfamide (Thrane *et al.,* 2000; De Souza *et al*., 2003a; Gross *et al.,* 2007; Kruijt *et al*., 2009; Van de Mortel *et al*., 2009).

A complicating factor on CLPs interaction studies is linked to the dual function of these molecules. In fact in addition to their direct effect on pathogen membranes, the surfactant property may enhance the exposition of the target pathogen to other antagonistic factors. The genetic and physiological mechanism involved in the cellular response of oomycetes and fungi remain unknown. It has been hypothesized that it could be related to the increasing of Ca and H ions influx in target cells, or that CLPs can interfere with canonical

signaling pathways by inhibiting the activity of a G-protein subunit (Raaijmmakers *et al.*, 2010).

1.2.6.3. Regulation of attachment and detachment to and from surfaces

Bacterial cells can attach to surfaces and, after cell division and proliferation, form dense aggregates commonly referred to as biofilms (rewieved by Raaijmakers *et al.* 2010). Polysaccharides and proteins secreted by bacterial cells form a hydrated gel-like slime that holds the biofilm together (Stewart and Franklin, 2008). Biofilm protects bacteria against adverse environmental conditions and protozoan predation and are a niche for horizontal gene transfer. Attachment and detachment of bacteria to surface is important in biofilm formation and CLPs play a role in this way, although the outcome may differ depending on the type of CLP (Raaijmakers *et al.*, 2010). Arthorfactin and putisolvins are involved in biofilm formation by *Pseudomonas* MIS38 and *P. putida* strains respectively, in fact an arthrofactin and putisolvin deficient mutant produce more bulky, but unstable, biofilms (Roongsawang *et al.*, 2003; Kuiper *et al.*, 2004; Kruijt *et al.*, 2009). However, for *P. fluorescens* strains SBW25 and SS101, viscosin and massetolide are pivotal for biofilm formation on a polystyrene surface, whereas CLP-deficient mutants formed substantially less biofilm (Raaijmakers *et al.*, 2010).

The different effect of CLP on biofilm formation is probably due to the diversity in structures and hydrophobicities of various CLPs, and it was suggested that depending on the cell surface charge of the producing strain and of the substratum, as well as the charge and hydrophobicity of the CLP (De Bruijn *et al.*, 2008). The ionic conditions and pH, can also influence the interaction of CLPs and other biosurfactants with a surface or interphase (Neu, 1996).

1.2.6.4. Motility

Movement of bacteria on surfaces has been extensively studied *in vitro* and several distinct forms have been recognized, including swimming, swarming and twitching (Henrichsen, 1972). During swimming and swarming movement, the flagella coalesce into a bundle, pushing the bacteria forward by rotating of the flagellar motor (Harshey, 2003). During swarming, vegetative cells of some bacterial species can differentiate into specialized swarmer cells and the viscosity of the surface can influence this cell differentiation (Harshey, 2003): for example, the presence of branched polymers to liquid medium, resulted in cell

differentiation in *Proteus mirabilis* and *Vibrio parahaemolyticus* (McCarter and Silverman, 1990; Allison *et al*., 1993). Biosurfactant property of CLPs can change the viscosity of surfaces, thereby influencing cell differentiation and motility. In plant-associated environments, biosurfactants may act as wettability agents and may promote not only cell motility but even solubilization and diffusion of substrates for growth (Lindow and Brandl, 2003).

Several studies carried out on CLP-deficient mutants have demonstrated that CLPs produced by antagonistic *Pseudomonas* strains and other bacteria are pivoltal for surface motility. Their surface motility was tested *in vitro* on semi-solid agar plates and in almost all cases, surface motility was lost in the mutant strains (Raaijmakers *et al*., 2010). Addition of the purified CLPs to the medium or of structurally related and unrelated biosurfactants can restore the reduced surface motility (Andersen *et al*., 2003; Kearns and Losick,2003; Kinsinger *et al*., 2003; De Bruijn *et al*., 2007).

The function of CLPs in natural habitats has not been studied in detail, but in *P. fluorescens* SS101, *P. fluorescens* 5064 and *Pseudomonas* DSS73 the colonization ability of the wild-type strain was more effective of the derivative CLP deficient mutant. In this context, Nielsen *et al*. (2005) suggested that CLPs help the producing bacteria to translocate more efficiently from an inoculum source to new and more nutrient-rich niches on the plant surface (Raaijmakers *et al*., 2010).

1.3. *Pseudomonas corrugata* and *Pseudomonas mediterranea*

1.3.1. Taxonomy and identification

P. corrugata and *P. mediterranea* are *γ-Proteobacteria* (Kersters *et al*., 1996), belonging to the genus *Pseudomonas sensu stricto* which includes species within rRNA similarity group I or the *fluorescens* rRNA branch (De Vos *et al*., 1985). Although this genus encompasses essentially fluorescent species, these two species as well as *P. sturtzeri* are non-fluorescent. Phylogenetic analysis based on 16S rRNA gene sequences placed *P. corrugata* within the *P. fluorescens* branch (Moore *et al* ., 1996; Anzai *et al* ., 2000), whereas in other studies on the basis of the combined nucleotide sequences of the *rpoD* and *gyrB* genes, it

was included in intrageneric cluster II within the '*P. fluorescens* complex' (*P. syringae* and *P. putida* being the other two) (Yamamoto *et al* ., 2000).

A *rpoB*-based phylogenetic tree showed that *P. corrugata* forms a cluster with *P. mediterranea* and *P. agarici* (Ait Tayeb *et al*., 2005). In addition, Cladera *et al*. (2006) analysed 16S rRNA, *rpoB*, *rpoD* and *gyrB* gene sequences of a number of *Pseudomonas* either independently or calculating a consensus matrix of the four genes. Based on the 16S rRNA gene only, *P. corrugata* and *P. mediterranea* formed a phylogenetic branch with recently described bacterial species composed of strains isolated from soil and/or plant rhizosphere, namely *P. kilonensis*, *P. brassicaceraum*, *P. thivervalensis*, *P. frederiksbergensis* and *Pseudomonas* strain OX1 (Cladera *et al*., 2006). These phylogenies were confirmed in a study with 107 *Pseudomonas* species. *P. corrugata* was defined as member of the *P. corrugata* subgroup of the *P. fluorescens* group within the *P. fluorescens* lineage. This group was constituted also by *P. mediterranea, P. kilonensis*, *P. thivervalensis* and *P. brassicacearum* (Mulet *et al*., 2010).

P. corrugata was described by Scarlett *et al.* (1978) as the causal agent of tomato pith necrosis (TPN), and further, but occasionally, also caused similar disease on chrysanthemum (Fiori, 1992), pepper (Lopez *et al*., 1994); and geranium (Magyarosy & Buchanan, 1995) (Fig. 8A). It has also been isolated from the root environment mainly from cultivated and bulk soil, roots and the rhizosphere of different plant species, in different countries (Kovacevich and Ryder, 1991; Ryder and Rovira, 1993; Schisler and Slininger, 1994; Catara *et al*., 1997; Scortichini, 1989; Achouak *et al*., 2000; Pandey *et al*., 2001; Walker *et al*., 2000).

Numerous studies outlined a high level of variability of phenotypic, chemotaxonomic and serological properties, and DNA-DNA hybridization data (Siverio *et al*., 1993, 1996; Catara *et al*., 1997; Sutra *et al*., 1997). In order to revise the taxonomic status of *P. corrugata*, Catara and collaborators (2002), used a polyphasic approach to study 27 strains representative of the various groups previously observed within *P. corrugata* (Catara *et al*., 1997; Sutra *et al*., 1997) by using numerical analysis of phenotypic tests, DNA±DNA hybridization, DNA fingerprinting techniques and 16S rDNA sequence analysis. Based on the biochemical characteristics the 27 strains were divided in two phena: the phenon A in which were included 19 strains and *P. corrugata* type strain CFBP 2431T; and the phenon B in which were clustered eight *P. corrugata* strains able to utilize *meso*-tartrate, 2-ketogluconate and histamine. In addition to this phenotypic data, DNA±DNA hybridization, PCR-based DNA typing methods applied to the whole genome (REP and BOX-PCR) or to selected genetic

regions (16S rDNA or ITS), and 16S rDNA sequence analysis, also delineated clearly two closely related, but distinct, taxa within the present *P. corrugata* species. Strains of phenon A, which contained the type strain CFBP 2431T, were defined as true *P. corrugata*, while strains included in phenon B were assigned to a novel species, named *Pseudomonas mediterranea*.

P. corrugata and *P. mediterranea* are similar Gram negative, non-spore forming rods. Both are strictly aerobic, do not produce fluorescent pigments, and are oxidase positive, reduce nitrates, do not produce levan, nor are they pectolytic. Colonies on YPGA (yeast peptone glucose agar) or nutrient dextrose agar are wrinkled, rarely smooth, and often produce yellow to brown diffusible pigments (Catara *et al.*, 2002; Sutra *et al.*, 1997) (Fig. 8B). Strains were reported to give variable results for arginine dihydrolase production and hypersensitivity response in tobacco leaf mesophyll (Catara *et al.*, 1997, 2002; Sutra *et al.*, 1997). Both species accumulate medium-chain-length polyhydroxyalkanoates (mcl-PHAs) like the majority of the *Pseudomonas* rRNA homology group I (Kessler and Palleroni, 2000; Solaiman *et al.*, 2000, 2005).

P. corrugata and *P. mediterranea* are phenotypically distinguishable on the ability of *P. mediterranea* to utilize histamine, 2-ketogluconate and *meso*-tartrate, whereas *P. corrugata* does not. *P. mediterranea* can also be clearly distinguished from *P. corrugata* by 16S rDNA analysis, by means of REP- and BOX-PCR profiles (Catara *et al.*, 2002); by using fingerprints generated by random-primed PCR (random amplified polymorphic DNA) (Catara *et al.*, 2000) and by using conventional PCR with two set of primers, type I primers PC5/1 and PC5/2 and type II primers PC1/1 and PC1/2) designed by Catara *et al.* (2000) which allow the identification and detection of *P. corrugata* and *P. mediterranea*, respectively. *P. mediterranea* strains were isolated from pith necrosis on tomatoes and peppers, mainly in France, Italy, Spain, Portugal and Turkey (Catara *et al.*, 2002; Basim *et al.*, 2005; Moura *et al.*, 2005; Sahin *et al.*, 2005).

I*n planta* test could not distinguish the two species based on pathogenicity or virulence. To screen tomato planting material reliably, Licciardello and collaborators (2011) developed a single tube quantitative real-time PCR assay, based on TaqMan chemistry with two primer pairs and TaqMan probes, differentially labeled, to detect and/or discriminate both bacterial pathogens. This method is field-transferable and provided a fast and sensitive tool for simultaneously detecting TPN causal agents and accurately identifying either *P. corrugata* or *P. mediterranea* and to assess the presence of mixed infections (Licciardello *et al.*, 2011).

1.3.2. Pathogencity and virulence

P. corrugata and *P. mediterranea* are the causal agent of Tomato Pith Necrosis (TPN). The characteristic symptom of the disease, as shown by its name, is stem pith necrosis and the pith may appear as: necrotic, dry and slightly disaggregated in the core; hydropic, white or dark but presenting a hard core and necrotic in the areas near the xylem; disaggregated due to the formation of ladder-like cavities (Fig. 8A). In an advanced stage of the disease the xylem becomes necrotic. The disease starts from the base of the stem and works up to the leaf stems and bunches. Necrosis can also affect the taproot and, occasionally, the rootlets.

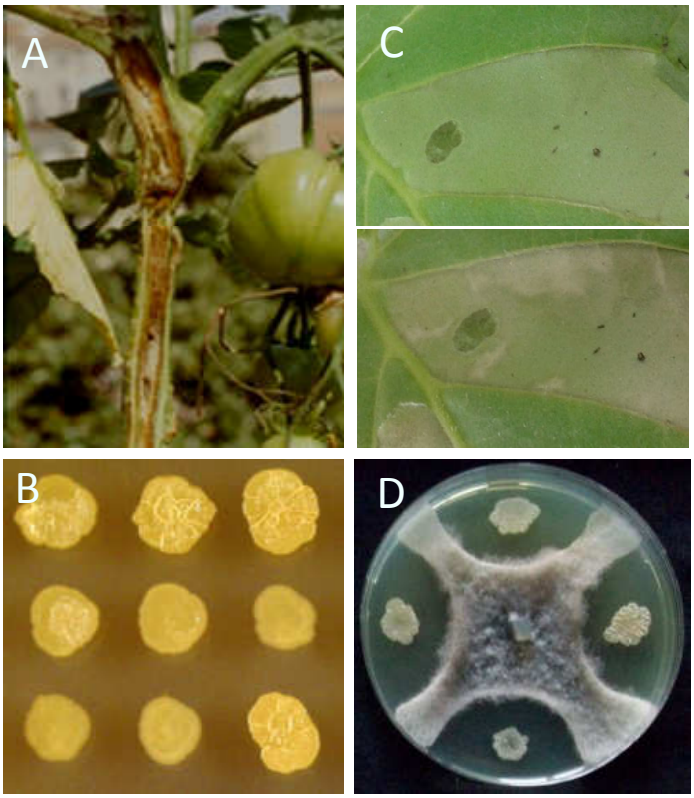


Fig. 8 – (A) Tomato stem showing typical TPN symptoms. (B) Colonies of *P. corrugata* and *P. mediterranea* on media containing glucose. (C) Hypersensitivity reaction on tobacco leaves 24 and 48 hour post inoculation with bacterial suspensions of *P. corrugata*. (D) Growth inhibiton of of *Botrytis cinerea* on potato dextrose agar induced by *P. corrugata* and *P. mediterranea*

Tomato plants artificially inoculated with a *P. corrugata* and a *P. mediterranea* strain showed reduced total tomato yield and fruit size (Moura *et al.*, 2005).

Some strains of *P. corrugata* are able to induce hypersensitive reaction (HR) when infiltrated in tobacco leaves (Catara *et al.*, 2007; Sutra *et al.*, 1997) (Fig. 8C) although the presence of a type III secretion system (TTSS) has not yet been ascertained. Live bacterial cells and *P. corrugata* culture liquid elicit phytoalexin (medicarpin) biosynthesis in white clover callus and HR in non-host tobacco leaves, K^+/H^+ exchange in tobacco leaf discs and transient formation of active oxygen species (hydrogen peroxide and superoxide) in clover callus cells (Gustine *et al.*, 1990, 1994, 1995). Data from Gustine *et al.* (1995) established that *P. corrugata* in culture produces at least two metabolites that apparently are responsible for these activities, HR1 and HR2. They are both peptides of less than 3500 kDa and either contained a chromophore or co-purified with a fluorescent compound (Gustine *et al.*, 1995). Activity of HR2, the primary component isolated from the water-soluble organic fraction, involved an increased pH in tobacco suspension cultures, K^+ efflux in tobacco leaf discs and elicitation of phytoalexin medicarpin biosynthesis (Gustine *et al.*, 1994, 1995). Elicitation of HR by HR2 cannot be ascribed to its functioning as a toxin because it did not cause TPN.

P. corrugata produces in culture phytotoxic and antimicrobial cationic cyclic lipopeptide (CLPs): corpeptins A and B and cormycin that will be thoroughly described below (Emanuele *et al.*, 1998 Scaloni *et al.*, 2004).

1.3.3. *P. corrugata* and *P. mediterranea* as biocontrol agents (BCA)

A number of *P. corrugata* strains were isolated from the root environment and were investigated as rhizosphere competent biocontrol agents (Catara, 2007). Moreover *in vitro* and *in vivo* activity against plant pathogenic fungi and bacteria was reported also for strains originally isolated as plant pathogens (Fig. 8D). The *in vitro* activity was reported against a number of *Bacillus* spp., *Clavibacter michiganensis* subsp. *michiganensis, Erwinia (Brenneria) quercina, Burkholderia cepacia, Pseudomonas syringae* pv. *pisi, P.s.* pv. *tomato, Agrobacterium tumefaciens* (Bell *et al.*, 1995; Chun and Leary, 1989; Cirvilleri *et al.*, 2001) as well as some phytopathogenic fungi: *Gaeumannomyces graminis* var. *tritici* (Ryder and Rovira, 1993), *Gibberella pulicaris* (Schisler and Slilinger, 1994), *Penicillium digitatum*, *Botrytis cinerea* (Cirvilleri *et al.*, 2000), *Sclerotinia sclerotiorum* (Fernando *et al.*, 2005), *Alternaria alternaria* and *Fusarium oxysporium* (Trivedi *et al.,* 2008) and against the chromista *Pythium aphanidermatum* (Zhou and Paulitz, 1993).

Table 1 - *Pseudomonas corrugata* as biocontrol agent: *in vivo* experiments under laboratory or field condition

Plant species	Disease	Pathogen	*P. corrugata* strain	Reference
Cucumber	Pythium root rot	*Pythium aphanidermatum*	13	Zhou e Paulitz, 1994; McCullagh *et al*., 1996
Cucumber	Damping off	*Pythium ultimum*	R 117	Georgakoupoulus *et al*., 2002
Grapevine	Postharvest - Grey mould	*Botrytis cinerea*	Various	Cirvilleri *et al*., 2001
Grapevine	Crown gall	*Agrobacterium tumefaciens*	Various	Bell *et al*., 1995
Lemon	Postharvest green mould	*Penicillium digitatum*	Various	Smilanick and Denis Arrue, 1992; Cirvilleri *et al*., 2001
Lemon	Malsecco	*Phoma tracheiphila*	Various	Coco *et al*., 2004
Maize	Damping off	*Pythium ultimum*	NRRL B-30409	Pandey *et al*., 2001
Maize	PGPR	-	NRRL B-30409	Trivedi *et al*., 2005
Nectarine, peaches	Postharvest brown rot	*Monilia fructicola*	Various	Smilanick *et al*., 1993
Pea	Root diseases	-	various	Chun *et al*., 1998
Potato	Silver scurf	*Helminthosporium solani*	Various	Chun and Shetty, 1994
Potato	Ring rot	*Clavibacter michiganensis subsp. sepedonicus*	Various	Schroeder and Chun 1995
Potato	Fusarium dry rot	*Giberella pulicaris*	Various	Schilsler *et al*. 1996
Potato	Wilt	*Verticillium dahliae*		Entry *et al*., 2000
Sugar beet	Pythium root rot	*Pythium ultimum*	2140luxAB	Schmidt *et al*., 1997 ; 2004
Sugar beet	Damping off	*Pythium ultimum*	R 117	Georgakoupoulus *et al*., 2002
Tomato	Fusarium wilt	*Fusarium oxysporum* f.sp. *lycopersici*	Various	Larkin and Fravel, 1998
Wheat	Take-all	*Gaemannomyces graminis* var. *tritici*	2140	Ryder e Rovira, 1993
Wheat	Take-all	*Gaeumannomyces graminis* var. *tritici*	2140R	Barnett *et al*., 1999 ; Ryder *et al*., 1999
Wheat	Rhizoctonia root rot	*Rhizoctonia solani*	2140R	Ryder *et al*., 1999
Wheat	Take-all	*Gaeumannomyces graminis* var. *tritici*	2140RlacZY	Choi *et al.*, 2003
Wheat	Pythium root rot	*Pythium spp.*	-	Chun and Gao, 1995
Tomato	Grey mildew	*B. cinerea*	P94	Guo *et al*., 2007

Some *P. corrugata* strains have been successfully tested as a biological control agent in different pathosystems (Table 1). *P. corrugata* strain 2140 was isolated from wheat field soil, and was reported that its rifampicin resistant derivative strain 2140R, colonize wheat roots after application on the seeds (Ryder and Borret, 1991), reduce take-all disease of wheat by *G. graminis* var. *tritici* (Barnett *et al*., 1998; Ryder *et al*., 1999) and *Pythium* root rot in sugarbeet (Schmidt *et al*., 2004). A mutant of this strain marked with a Tn7-derived

transposon containing *lac*ZY, it has been authorized for field release in Australia (Ryder *et al.*, 1994), and did not reveal any major changes to the lacZY insert site and any polymorphism differences between the introduced strain and soil re-isolates (Choi *et al.*, 2003). A *lux*AB mutant of the same strain (*2140lux*AB) has been employed in a commercial formulation of seed pellet of sugar beet toward *Pythium ultimum*. In these tests 10^4 to 10^6 CFU per seed resulted in maximal survival of plants, whereas increasing the antagonist inoculum density, the survival and dry weight of sugar beet decreased (Schmidt *et al.*, 2004). *P. corrugata* strain 13 selected amongst bacteria trapped in soil by cucumber roots reduced *P. aphanidermatum* zoospore attraction and germination of encysted zoospores in cucumber root exudates from bacteria-treated plants (Zhou and Paulitz, 1993). In cucumbers grown in greenhouse in rock wool system, *P. corrugata* strain 13 reduced disease severity under high disease levels and increased yields in the absence of the pathogen of thus suggesting a plant growth-promoting (PGPR) effect (McCullagh *et al.*, 1996). Evidence of induced resistance has been observed in the same pathosystem after inoculating the pathogen and the *P. corrugata* strain in the spatially separated root system where stem rot was delayed and disease index reduced (Zhou and Paulitz, 1994). Furthermore, a correlation has been observed between resistance induction and salicylic acid production (Chen *et al.*, 1999).

A *P. corrugata* strain NRRL B-30409 a soil bacterium originally isolated from a temperate site of Indian Himalayan Region (IHR) was examined for its antagonistic activities against two phytopathogenic fungi, *Alternaria alternata* and *Fusarium oxysporum* (Pandey and Palni, 1998; Kumar *et al.*, 2007; Trivedi *et al.*, 2008). The antagonism was affected by growth medium, pH and temperature. Maximum reduction in biomass, of *A. alternata* and *F. oxysporum*, was observed at pH 5.5 and 21°C. The production of siderophore, ammonia, lipase and chitinase in growth medium by *P. corrugata* were considered contributing to the antagonistic activities of the bacterium. *P. corrugata* strain NRRL B-30409 did not produce HCN *in vitro* whereas ammonia production was detected (Trivedi *et al.*, 2008).

Two patents have been deposited in the US for the use of *P. corrugata* as a biological control agent (Chun, 2000; US Patent no. 6,156,560) and to select desiccation tolerant strains of bacteria (Chun, 2002; US Patent no. 6,383,798).

P. corrugata's efficacy as a biological control agent seems linked to its elevated rhizosphere competence. In addition, its *in vitro* antimicrobial activity against a long list of microorganisms (Gram-negative and Gram-positive bacteria, Chromista, yeast, fungi) shows it is able to compete by producing substances possessing antimicrobial activity and probably

also by competing for iron through the siderophore corrugatin (Meyer *et al.*, 2002; Risse *et al.*, 1998). Antimicrobial activity could be the result of the production CLPs (Emanuele *et al.*, 1998; Scaloni *et al.*, 2004) and of other substances, as suggested by data obtained from research studies or sequences deposited in the GenBank: inhibitory volatiles (Fernando *et al.*, 2005), hydrogen cyanide (Ramette *et al.*, 2003), 2,4-diacetylphluoroglucinol (GenBank no. AJ515694, AJ515693, AJ515692), pyrrolnitrin (Garbeva *et al.*, 2001).

1.3.4. Poly(hydroxyalkanoates) (PHAs) production

Poly(hydroxyalkanoates) (PHAs), are microbial polyesters synthesized by a variety of bacteria. These biodegradable polymers are accumulated as inclusion bodies when nutrient supplies are imbalanced and are thus believed to play a role in bacteria establishment, proliferation, survival and competition, especially in competitive environments where carbon and energy sources are limiting, such as those encountered in the soil and rhizosphere (Kadouri *et al.*, 2005; Solaiman *et al.*, 2005) (Fig. 9). PHAs have great potential for industrial and medical applications, to serve as ecologically sound substitutes for petroleum-derived polymers because they are biodegradable and can be produced by white-biotechnological process using renewable (or low-cost) feedstocks. As such, research abounds to explore the use of PHAs in medical devices, foods, agriculture and consumer products (Zinn *et al.*, 2001; Snell and Peoples, 2002; Lenz and Marchessault, 2005).

There are three major groups of PHAs based on their repeat unit compositions: the short-chain-length (scl-) PHAs where the repeat units are hydroxy fatty acids (HFAs) of 3–5 carbon chain length (C3–C5), the medium-chain-length (mcl-) PHAs with repeat units of C6 and longer, and the scl-*co*-mcl-PHAs such as NODAXTM (Procter & Gamble, Cincinnati, OH, USA) that contain primarily 3-hydroxybutyrate but also some ≥ C6 HFAs as their repeat units. In general, the scl and scl-*co*-mcl-PHAs are thermoplastics with various degrees of crystallinity, and the mcl-PHAs are amorphous and have elastomeric and adhesive properties.

It was demonstrated that *P. corrugata* accumulates medium-chain-length polyhydroxyalkanoates (mcl-PHAs) like the majority of the *Pseudomonas* rRNA homology group I (Kessler and Palleroni, 2000; Solaiman *et al.*, 2000, 2005).

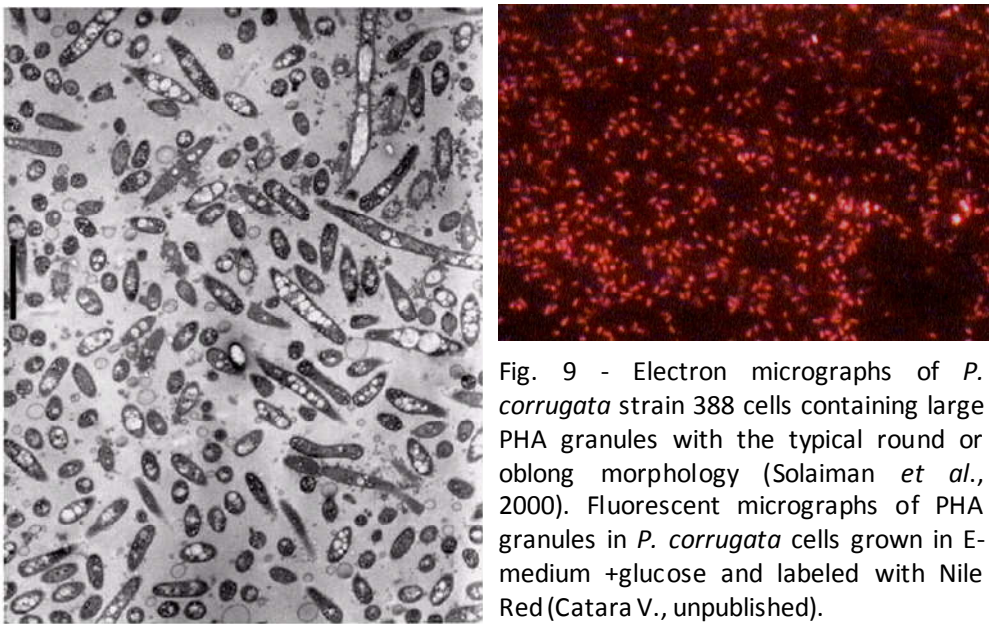


Fig. 9 - Electron micrographs of *P. corrugata* strain 388 cells containing large PHA granules with the typical round or oblong morphology (Solaiman *et al.*, 2000). Fluorescent micrographs of PHA granules in *P. corrugata* cells grown in E-medium +glucose and labeled with Nile Red (Catara V., unpublished).

P. corrugata PHA composition was first investigated on strain 388 isolated as endophyte in alfalfa roots. The mcl-PHAs synthesized by this bacterium contain β-hydroxydecanoate (C_{10}), β-hydroxyoctanoate (C_8), and β-hydroxytetradecenoate ($C_{14:1}$) as their major repeat-unit monomers (Solaiman *et al.*, 2000). Later the composition of strain IPVCT 10.2 and of *P. mediterranea* strain CFBP 5447 was investigated. Their composition was similar to strain 388, although showed lower level of C_8-monomer and higher level of C_{10}-monomer than that found in strain 388 (Solaiman *et al.*, 2005). It is noteworthy that *P. corrugata* can produce mcl-PHAs not only from pure sources, such as very expensive long-chain fatty acids, but also from renewable (or low-cost) sources such as biodiesel (Ashby *et al.*, 2003), glycerol (Ashby *et al.*, 2005), used cooking oils (V. Catara, unpublished results) and soy molassa (Solaiman *et al.*, 2006). Recently *P. mediterranea* CFBP 54547 PHA production from refined and crude glycerol was investigated and it was discovered that best rate of PHA was obtained when 2% of crude glycerol was supplied (Palmeri *et al.*, 2012).

P. corrugata have a type II PHA genetic system consisting of two synthase genes (*phaC1, phaC2*) separated by a gene encoding for the depolymerization of PHA (*phaZ*). This genetic organization is commonly found in mcl-PHA-producing pseudomonads.

Using PCR methods that specifically amplify segments of medium-chain-length poly(hydroxyalkanoate) (mcl-PHA) synthase genes (*phaC1* and *phaC2*), Solaiman and collaborators (2005), screened a collection of *P. corrugata* and *P. mediterranea* strains in

order to evaluate for the presence and variability of *pha* loci, demonstrating that *P. mediterranea* also produce PHA.

Conte *et al.*, (2006) examined the polyhydroxyalkanoate (PHA) synthases *phaC1* and *phaC2* gene expression in two strains of *P. corrugata* grown in a minimum mineral medium with related (oleic acid and octanoate) or unrelated (glucose) carbon sources. Transcriptional analysis revealed that no polycistronic transcript was expressed under any culture conditions indicating that *phaC1* and *phaC2* are not cotranscribed in *P. corrugata*, as reported for other pseudomonads (Hoffmann and Rehm 2004; 2005). The authors also showed that each PHA synthase was differentially expressed depending on the carbon source utilized for bacterial growth. *phaC1* was upregulated in cultures grown with oleic acid, whereas in cultures with glucose or sodium octanoate, both *phaC1* and *phaC2* were induced but at different rates. They also demonstrated that exist a significant correlation between PHA production and *phaC1*/*phaC2* expression. This differential expression suggested that at least two distinct networks exist for the regulation of *phaC1* and *phaC2* and that probably a putative promoter(s) is present upstream of *phaC2* also in *P. corrugata*.

Further studies carried out by Solaiman and collaborators (2008) described and characterized the genetic organization of the *pha* locus of *P. corrugata* strain 388, and reported the first manipulation of the expression pattern of PHA synthase genes, the mcl-PHA composition, and the PHA granule morphology through the removal of an intergenic region of the *pha* locus. The BLASTN analysis of entire *pha* locus (*phaC1-phaZ-phaC2)* of *P. corrugata* 388 showed the best sequence match with the *pha* loci of *P. corrugata* strain CFBP 5454 (AY910767) and *P. mediterranea* strain CFBP 5447 (AY910768). The PHA synthases and depolymerases of the two *P. corrugata* strains (388, CFBP 5454), at both the nucleotide and amino-acid levels, are nearly identical. The three *pha* genes of *P. corrugata* 388 showed high homology to those of *P. mediterranea* CFBP5447, however a significant difference was observed in the *phaC1–phaZ* intergenic regions of the two phylogenically related strains. The *phaC1–phaZ* intergenic region of *P. corrugata* 388 in fact contains a strong hairpin structure that is predicted to be a putative intrinsic transcription terminator for the *phaC1* gene, consisting of a dyad symmetry (24 bp; ΔG = - 41.8 kcals). This structure is in effect absent in the much shorter *phaC1– phaZ* intergenic sequence of *P. mediterranea* CFBP 5447 (Solaiman *et al.,* 2008).

P. corrugata mutant-clones (XI 32-1 and XI 32-4) in which this intergenic region was replaced with a selectable kanamycin-resistance gene were grown on oleic acid for 48 h and

showed a significant increases of *phaC1* and *phaC2* relative expression in comparison to the initial inoculants, whereas the parental strain exhibited only a slight induction of transcripts. Furthermore, in comparison to parental *P. corrugata* with only a few large PHA inclusion bodies, the mutants grown on oleic acid produce numerous smaller PHA granules that line the periphery of the cells, whereas with glucose as a substrate, mutants produced mcl-PHA with a high content of the monounsaturated 3-hydroxydodecenoate as a repeat-unit monomer, demonstrating for the first time the *phaC1–phaZ* intergenic region possess effect on the substrate-dependent temporal expression of *phaC1* and *phaC2* genes, the repeat unit composition of mcl-PHA, and the morphology of the PHA granules (Solaiman *et al.*, 2008).

1.3.5. *P. corrugata* Cyclic Lipopeptides (CLPs)

P. corrugata produces two kinds of cyclic lipopeptides: corpeptins (Emanuele *et al.,* 1997) and cormycin A (Scaloni *et al.,* 2004) (Fig. 10).

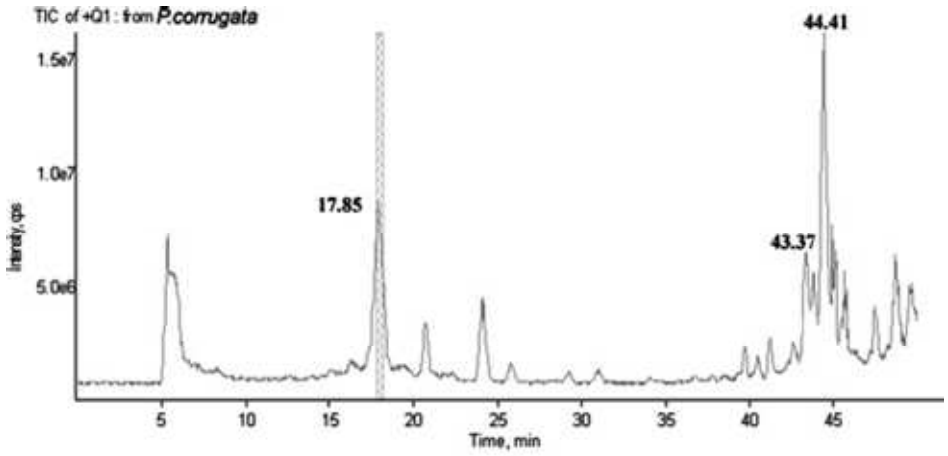


Fig. 10 - Representative chromatogram of *P. corrugata* PVCT 10.3 culture filtrate. The profile reports peaks eluting at 17.85 min corresponding to Cormycin-A and two peaks at 43.37 and 44.41 min corresponding to corpeptin A and B. (Scaloni *et al.*, 2004)

Corpeptins (CPs) were isolated from the culture filtrates of a *P. corrugata* strain NCPPB 2445 isolated from tomato. In order of their appearance in the reverse-phase HPLC eluate, they were named corpeptin A (CP-A) and corpeptin B (CP-B). Their molecular weights

(MH^+ 2094 and 2120, respectively for CP-A and CP-B) were compatible with those of other previously described peptins. The combined use of fast atom bombardment-mass spectrometry (FAB-MS), nuclear magnetic resonance (NMR) spectroscopy and chemical procedures determined the following primary structure to the peptide moiety: Dhb-Pro-Ala-Ala-Ala-Val-Val-Dhb-Hse-Val-aIle-Dhp-Ala-Ala-Ala-Val-Dhb-aThr-Ala-Dab-Set-Ile with the terminal carboxy group closing a macrocyclic ring on the hydroxy group of the allo-threonine residue. The CPs peptide moiety compared to that of other *Pseudomonas* peptins shows a very high homology among CPs, syringopeptins (SP22s and SP25s) and fuscopeptins (FPs), somehow smaller but still noticeable in tolaasins (ToI-A). 2D-NMR revealed that the N-terminus is in turn acylated by 3-hydroxydecanoate in CP-A and by *cis*-3-hydroxy-5-dodecenoate in CP-B. The fatty acid moiety of CP-A is the same found in SP22-A,SP25-A and FP-B while that of CP-B includes an unsaturation. Preliminary data on the phytotoxic activity of CPs' indicate that have properties partly different from those of SPs and FPs. In the tobacco leaf assay induced chlorosis 72 h after tissue infiltration whereas a high intensity of necrotic symptoms was induced by the two SPs at the same concentration. The same assay also displayed that CP-A possess more phyotoxic activity against tobacco than CP-B. The antimicrobial activities of the CPs is instead very similar to those of SPs showing inhibitory activity only against *Bacillus megaterium.*

No nonapeptides were found in the culture filtrate of this strain although is known that *Pseudomonas* strains usually produce both groups of CLPs. This unexpected finding prompted Scaloni and collaborators (2004) to screen culture filtrates of several *P. corrugata* wild-type strains and their chromatographic analysis revealed that nonapeptide production is strain-dependent and characterized a novel compound.

Cormycin A (CM-A) was isolated from the culture filtrate of *P. corrugata* strain IPVCT 10.3. The complete structure of CM-A was elucidated by combined chemical degradation, MS and two-dimensional NMR procedures. Its peptide moiety corresponds to L-Ser-D-Orn-L-Asn-D-Hse-L-His-L-*a*Thr-Z-Dhb-L-Asp(3-OH)-L-Thr(4-Cl) with the terminal carboxy group closing a macrocyclic ring with the hydroxy group of the N-terminal serine residue. This is, in turn, N-acylated by 3,4-dihydroxy-esadecanoate. *P. corrugata* culture filtrates as well as low micromolar concentrations of purified CM-A presented a strong antimicrobial activity against *B. megaterium* and *R. pilimanae.* CM-A activity against the two sensitive microorganisms was higher than that observed for other cyclic nonapeptides syringomycin E (SR-E) and syringotoxin (ST) from *P. syringae*. In addition, *P. corrugata* culture filtrates and purified CM-A

also exhibited phytotoxic activity inducing chlorosis and after 48h similar necrotic symptoms on *Nicotiana tabacum* leaves, thus confirming that this peptide is essential for the biological activity of this bacterium. Also in this case CM-A phytotoxic activity was higher than that observed for SR-E and ST. CM-A lysed human, rabbit and sheep red blood cells (RBCs) and its haemolytic activity was concentration dependent. It was similar with human and rabbit erythrocytes, but 5-fold smaller with sheep erythrocytes. A similar trend was observed for SR-E and ST although comparative measurements showed that also in this case CM-A is more active (Dalla Serra *et al.*, 1999; 2003). CM-A was also able to interact with and permeabilize artificial membranes (liposomes). Peptide concentration and liposome lipid composition seem to be pivotal for CM-A membrane interaction. CM-A showed major activity in presence of cholesterol (mainly present in animal cell membranes) followed by ergosterol (a major component of fungal plasma membranes) and stigmasterol (the main sterol component of higher plant membranes). This preference for sterol-containing membranes has been already reported for SR-E (Dalla Serra *et al.*, 1999) and ST, although animal cells are not the natural targets of these CLPs. The presence of negatively charged lipids (i.e. phosphatidic acid and phosphatidylinositol) did not improve, but rather hampered, CM-A activity. The reduced CM-A permeabilization activity on liposomes containing stigmasterol correlates well with the lower haemolytic activity measured on sheep RBCs, which present a higher content of this sterol and a reduced percentage of phosphatidylcholine with respect to the other RBCs tested.

The pore-forming mechanism of action for CM-A was supposed and to better understand the strongest interaction of CM-A with RBCs, the critical micellar concentration (MIC) of this peptide was measured. Normally, this parameter is inversely related to membrane permeabilizing properties. CM-A presents a critical micellar concentration lower than that measured for SR-E (138 μM) (Dalla Serra *et al.*, 1999), accordingly, to the more hydrophobicity than SR-E, and consistent with the strongest propensity of CM-A to partition into membranes. The pore-forming mechanism of action for CM-A was also well supported by planar lipid-bilayer experiments, where addition of CM-A to a solution bathing a planar lipid bilayer (7:3 (mol/mol) 1,2-diphytanoyl-*sn*-glycerophosphocholine/cholesterol) increased the membrane conductance, causing step-like current transitions. This behaviour is peculiar to the formation of structured conducting pathways across the membrane (i.e. pores), rather than being due to an unspecific detergent-like activity. Preliminary modelling studies on CM-A also suggested that this CLP should form oligomeric channel structures containing six to

eight monomers (results not shown), in agreement with biological data derived from lipid bilayer permeabilization measurements (Scaloni *et al.,* 2004).

1.3.6. The Acyl-homoserine lactone (AHL) Quorum Sensing (QS) system

1.3.6.1 Bacteria cell-cell communication and the paradigm of QS

In the last 20 years, microbiologists' interest focused on bacteria major level of gene regulation, which involves intercellular communication via the production and response to signal molecules. These molecules represent the languages by which bacteria communicate and respond to environmental stimuli by the activation or repression of specific target genes. The concentration of the signal molecules produced by cells increase concomitantly with bacterial population density, and upon reaching a critical concentration, results in the alteration of target genes expression. This cell-density-dependent mechanism of gene regulation has been termed Quorum Sensing (QS) (Fuqua *et al*., 1994). The paradigm of QS, hystorically known as autoinduction, was first discovered and described in the bioluminescent marine bacterium *Vibrio fischeri* (Fuqua *et al*., 1994). *V. fischeri* is a symbiotic bacterium which produce light when colonizing the light organs of certain marine animals. The autoinduction is an environmental sensing system which allows bacteria to monitor their own population density. Bacteria produce diffusible compounds, the autoinducer, which accumulate in the surrounding environment during growth. At low cell densities this substance is in low concentration, but when bacteria achieve high cell densities, this substance accumulates to the critical concentration (*quorum*) required for luminescence genes activation (Fuqua *et al*., 1994).

Two proteins belonging to the LuxI-LuxR protein families control the expression of the *lux*ICDABEG operon involved in regulating light production in *V. fischeri*. The LuxI-type protein synthesize the autoinducer *N*-(3-oxo-hexanoyl)-L-homoserine-lactone (3-oxo-C_6-AHL), whereas the LuxR is the AHL-response regulator. The signal molecules diffuse across the bacterial membrane and inside the cell interact directly with the cognate LuxR receptor. The AHL-LuxR active complex binds the palindromic *lux* box element located in the promoter region of the *lux* regulon. The *luxI* and *luxR* themselves are part of this regulon and are therefore regulated by a positive loop resulting in the signal amplification (Fuqua *et al.*,1994,

Fuqua *et al.*, 1996; Stevens and Greenberg, 1997; Miller and Bassler, 2001)). Thus, based on this paradigm, the AHL-QS system described in other bacteria consist of two elements, an AHL synthase, encoded by *luxI* homologs genes and a receptor/transcriptional activator protein encoded by *luxR* homologs genes. The AHL-receptor complex recognize the *lux* box element in the promoter region of specific target genes regulating their expression (Whitehead *et al.*, 2001). Cells express the AHL-synthase at a basal level when in low population densities, so AHL concentration is low. However, as the population density increases, so does the concentration of AHL in the environment. LuxR-type proteins show high specify for their cognate signal molecule, guaranteeing a good degree of selectivity, although, it has been demonstrated that LuxR-type proteins can also interact with AHLs of different length and substitution, and from different species of bacteria, raising important implications for the role of AHLs in interspecies communication. This ability to "listen" other QS signal represent an important aspect of interpopulation communication and community structuring in natural environments (Bassler,2002) .

Several chemical classes of signal molecules have now been identified. Gram-positive signals are usually modified peptides (Bassler,2002); whereas many Gram-negative bacteria use *N*-acyl homoserine lactones (AHLs) as QS signal molecules. Other types of signal molecules have also been identified such as the autoinducer AI-2 (Chen *et al.*, 2002), the diffusible signal factor (DSF) produced by *Xanthomonas campestris* (Barber *et al.*, 1997), and quinolones produced by *P. aeruginosa* (Pesci and Iglewski, 1999). In particular, AHLs are diffusible signal molecules which consist of a variable acyl chain attached to a conserved homoserine lactone head group. Specificity is conferred by the length of the acyl chain (short, 4-8 carbons; or long, 10-18 carbons), the nature of the substitution at carbon 3 (oxo- or hydroxyl- group substitution) and the presence of a double bond.

The presence of AHLs, and consequently of several AHL QS systems, is mainly detected by using bacterial biosensors. These biosensors are strains unable to produce AHLs (lack the AHL synthase enzyme) and contain a functional LuxR protein cloned upstream a cognate target promoter (usually the promoter of the cognate *luxI* synthase), which positively regulates the transcription of a reporter gene in presence of exogenous AHLs (e.g. bioluminescence, β-galactosidase, green-fluorescent protein and violacein pigment production) (Steindler and Venturi, 2007).

1.3.6.2. The AHL-QS in plant associated *Pseudomonas*

Bacterial QS has been described in Gram-positive and Gram-negative bacteria and it has been demonstrated that a wide range of behaviors are regulated by this system, including bioluminescence, sporulation, cell division, biofilm formation, swarming, swimming, mating, competition, stress survival, and colonization and virulence factor production, such as extracellular polysaccharides (EPS), surfactants, antibiotic and extracellular enzymes, antibiotic biosynthesis (Fuqua *et al.*, 2001; Whitehead *et al.*, 2001; Withers *et al.,* 2001).

In plant pathogenic bacteria, QS mediated by AHLs has been observed intimately linked to the control of several virulence factors such as exoenzyme production in *Erwinia carotovora* subspecies, extracellular polysaccharides in *Pantoea stewartii* ssp. *stewartii*, toxin production in *Burkolderia glumae*, Ti plasmid conjugal transfer system in *Agrobacterium tumefaciens* and traits involved in survival, host invasion and virulence in *P. syringae* pv. *syrinage* (Barnard and Salmond 2007; Dulla and Lindow 2008; Kim *et al.*, 2004; Quinones *et al.* 2004, 2005; von Bodman and Farrand 1995; von Bodman *et al.* 2003; Whitehead *et al.* 2001; Zhang *et al.* 1993).

Pseudomonads are studied for their roles as plant pathogens, for their ability to colonize plant-related niches, such as the rhizosphere and as human opportunistic pathogens (*P. aeruginosa*) (Venturi, 2006; Wei & Zhang, 2006). A list of the AHL QS systems identified in plant associated and phytopathogenic *Pseudomonas* is summarized in Table 2 and Table 3 respectively. The AHL-QS system that has been most extensively studied is the *P. aeruginosa*. This bacterium presents two systems, the LasI/R and RhlI/R producing and responding to 3-oxo-C_{12}-AHL and C_4-AHL respectively (Juhas *et al.*, 2005; Smith and Iglewski, 2003). Several virulence factor has been found to be regulated by both systems, including elastase, alkaline protease, exotoxin A, rhamnolipids, pyocyanin, lectins, superoxide dismutase and biofilm formation. Regarding plant associated *Pseudomonas* AHL-QS has been shown to regulate traits involved in biological control activity against plant pathogens by the synthesis of secondary metabolites.

One of the first reports concerned the AHL-QS in *Pseudomonas* was the PhzI/R system of the wheat-plant growth-promoting rhizosphere-colonizing *P. aureofaciens* strain 30-84 (Pierson *et al.*, 1994, 1995; Wood *et al.,* 1997). PhzI directs the synthesis of C_6-AHL, which interacts with cognate regulator PhzR, bind the *lux*-box like element in the promoter region of the phenazine operon *phzXYFABCD* , regulating its transcription. A second AHL-QS

system, designated CsaI/R, was also found in *P. aureofaciens* 30-84 (Zhang & Pierson, 2001). This system is not involved in phenazine regulation, whereas it regulates rhizosphere competitiveness, exoprotease production and cell-surface component biosynthesis. Among the biocontrol *P. fluorescens* strains, the *P. fluorescens* 2-79 PhzI/R (C_6-C_8-AHL; 3-OH-C_6-C_7-C_8-C_{10}-AHL; 3-oxo-C_6-AHL) system has been reported to be involved in the regulation of the antifungal metabolite phenazine-1-carboxamide production (Khan *et al.*, 2005). Laue *et al.*, (2000) showed that the rhizosphere-colonizing biocontrol strain *P. fluorescens* F113 possess a non canonical AHL-QS system. It, in fact, produce three AHL molecules (C_6-C_{10}-AHL and 3-OH-$C_{14:1}$-AHL) through a novel AHL synthase termed HdtS. This synthase potentially represents a new family, as it does not belong to either of the known AHL synthase families (LuxI and LuxM). However the response regulator and phenotype regualated have not yet been investigated. AHL-QS systems have been studied from beneficial rhizosphere *P. putida.* Strains WCS358, IsoF and PCL1445 share orthologous systems designated PpuI/R, responsible for the synthesis and response mainly to 3-oxo-C_{12}-AHL. The PpuI/R system of strain IsoF has been shown to influence structural biofilm development, whereas in strain PCL 1445 seems to regulate biofilm and biosurfactant production, and CLP putisolvins biosynthesis. No PpuI/R-regulated phenotype has yet been found for strain WCS358 (Steidle *et al.*, 2002; Bertani and Venturi, 2004; Dubern *et al.*, 2006).

AHL-QS system has been reported is the plant pathogen *P. syringae* pv. *syringae*, which is the causal agent of brown spot in bean (Dumenyo *et al.*, 1998; Quinones *et al.*, 2004). *Pseudomonas syringae* B728a AHL-QS system AhlI/R synthesizes and respond to 3-oxo-C_6-AHL. This system has been shown to be important for cell-aggregation and epiphytic fitness for *in planta* growth and disease (Quinones *et al.*, 2004). *Pseudomonas syringae* pv. *tabaci* 6605, is a phytopathogenic bacterial isolate, causal agent of the wildfire disease on tobacco. Its PsyI/R (C_6-C_8-AHL; 3-oxo-C_6-AHL) has been demonstrated to be involved in exopolysaccharide (EPS) and siderophore production, iron up-take, biofilm formation, H_2O_2 and antibiotic tolerance and virulence on tobacco (Taguchi *et al.,* 2006). Recently, the PssI/R system (3-oxo-C_6-C_8-AHL) of *P. savastanoi* pv. *savastanoi* DAPPG-722 causal agent of the knot disease of olive tree has been described. The authors investigated several phenotypic traits for AHL-QS regulation, and reported that only EPS and protease production and virulence *in planta* are regulated by the PssI/R system in *P. savastanoi*; however it was not clear if EPS is involved in the disease process (Hosni *et al.*, 2011). *P. fuscovaginae* possesses

Table 2 - AHL production and regulated phenotypes in plant-associated *Pseudomonas*

Species/Strain	Relevant characteristics	AHL-QS system	AHL molecules	QS regulated phenotype(s)	Reference
P. putida					
PCL 1445	Colonizes grass roots and produces biosurfactants	PpuI/R	3-oxo-C_6-C_8-C_{10}-C_{12}-AHL	Putisolvins production, Biofilm formation, Biosurfactant production	Dubern *et al.*,2006
WCS358	PGPR isolated from potato roots.	PpuI/R	3-oxo-C_6-C_8-C_{10}-C_{12}-AHL	Currently unknown	Bertani and Venturi, 2004
IsoF	PGPR isolated from tomato roots	PpuI/R	3-oxo-C_6-C_8-C_{10}-C_{12}-AHL	Biofilm formation	Steidle *et al.*, 2002
P. aureofaciens 30-84	Wheat PGPR	PhzI/R CsaI/R	C_6-AHL Currently unknown	Phenazine production Rhizosphere competitiveness, Cell-surface component biosynthesis, Exoprotease production	Pierson *et al.*, 1994; Wood *et al.*,1997; Zhang and Pierson, 2001
P.chlororaphis PLC 1391	Plant beneficial rhizobacterium	PhzI/R	C_6-AHL	Phenazine-1-carboxamide production	Chin *et al.*, 2001, 2005
P.fluorescens					
2-79	PGPR	PhzI/R	C_6-C_8-AHL 3-OH-C_6-C_7-C_8-C_{10}-AHL 3-oxo-C_6-AHL	Phenazine-1-carboxamide production	Khan *et al.*, 2005
NCIMB 10568	Soil-borne bacterium	MupI/R	3-oxo-C_{10}-AHL	Mupirocin biosynthesis	Sayed *et al.*,2001; Hothersall *et al.*, 2011
F113	Biological control, 2,4-diacetylphloroglucinol producer	HdtS	C_6-C_{10}-AHL 3-OH-$C_{14:1}$-AHL	Currently unknown	Laue *et al.*,2000
2P24	Biological control	PcoI/R	C_6-C_8-AHL 3-oxo-C_6-C_8-AHL	Biofilm formation, Colonization on wheat rhizosphere, Biocontrol ability	Wei and Zhang 2006,
P. brassicacearum NFM 421	Plant-beneficial	Currently unknown	C_{14}-AHL 3-OH-C_{10}-C_{14}-AHL	Currently unknown	Lalaouna *et al.*,2012

Table 3 - AHL production and regulated phenotypes phytopathogenic *Pseudomonas*

Species/Strain	Relevant characteristics	AHL-QS system	AHL molecules	QS regulated phenotype(s)	Reference
P. corrugata CFBP 5454	Causal agent of tomato pith necrosis	PcoI/R	C_6-C_8-AHL 3-oxo-C_6-AHL	CLP production, Colony morphology, Virulence, Biosurfactant activity, Antimicrobial activity, Swarming motility, HR	Licciardello *et al*., 2007, 2012
P. mediterranea CFBP 5447	Causal agent of tomato pith necrosis	PmeI/R	C_6-C_8-AHL 3-oxo-C_6-AHL	CLP production, Colony morphology, Virulence, Biosurfactant activity, Antimicrobial activity	Licciardello *et al*.,2012
P. syringae					
pv. *syringae* B728a	Causal agent of brown spot of bean	AhlI/R	3-oxo-C_6-AHL	Cell aggregation Epiphytic fitness for in planta growth and disease. EPS production, Colony morphology, Alginate production, Swarming motility, H_2O_2 tolerance, Virulence.	Quinones *et al*., 2004; 2005
pv. *tabaci* 6605	Causal agent of wildfire disease on host tobacco plants. Induces hypersensitive reaction (HR) on nonhost plants.	PsyI/R	C_6-C_8-AHL; 3-oxo-C_6-AHL	EPS production, Virulence on tobacco, Iron up-take, Biofilm formation, Siderophore production, H_2O_2 and antibiotic tolerance.	Taguchi *et al*., 2006
P. fuscovaginae UPB0736	pathogen of rice and causes bacterial brown sheath rot on several cereals. UPB0736 isolated from diseased rice in Madagascar	PfsI/R PfvI/R	3-oxo-C_{10}-C_{12}-AHL C_{10}-C_{12}-AHL	Plant pathogenicity	Mattiuzzo *et al*., 2010
P. savastanoi pv. *savastanoi* DAPPG-722	Olive knots (Italy)	PssI/R	3-oxo-C_6-C_8-AHL	Virulence, EPS production	Hosni *et al*., 2011

two conserved AHL-QS systems designated PfsI/R and PfvI/R. The PfsI/R produces and responds to C_{10}-AHL and C_{12}-AHL whereas PfvI/R produces several long-chain 3-oxo-AHLs and responds to 3-oxo-C_{10}-AHL and 3-oxo-C_{12}-AHL and at high AHL concentrations can also respond to structurally different long-chain AHLs. The *pfsI/R* system was regulated by a novel repressor RsaM, while the *pfvI/R* system was regulated by both the RsaL repressor and by RsaM. The two systems are not transcriptionally hierarchically organized but share a common AHL response and both are required for plant virulence. No phenotypes have been yet investigated (Mattiuzzo *et al.*, 2010).

1.3.6.3. *Pseudomonas corrugata* AHL QS system

P. corrugata has been reported as potentially being able to produce N-acyl homoserine lactones (AHLs) as inter-cellular signal molecules (Dumenyo *et al.*, 1998; Elasri *et al.*, 2001). Licciardello *et al.*, (2007) demonstrated, for the first time, that *P. corrugata* possess a very well conserved AHL-QS system involved in the regulation of microbiological and phytopathogenic traits of this bacterium. Testing a representative collection of strains of *P. corrugata*,for the AHL production, isolated from various and distinct geographic regions, from diverse plant species or from soil and having different characteristics (i.e. nutritional, fatty acid and DNA-fingerprinting profiles), the authors demonstrated that despite their heterogeneous phenotypic and genotypic characteristics, all the strains produce comparable levels of the same AHLs: C_6-AHL, C_6-3-oxo-AHL and C_8-AHL, with C_6-AHL apparently being the most abundantly signal molecule produced.

The screening of a genomic library of a strain of *P. corrugata* (CFBP 5454) allowed the identification in a cosmid insert (pLC3.34) of the genetic determinants encoding for the AHL-QS designated PcoI/PcoR, homologous to members of LuxI and LuxR family proteins respectively. Analysis of the *pcoI* promoter region, revealed a 20-bp imperfect palindrome sequence located 67 bp upstream of the putative translational start site, having high similarity to *lux* box-like elements of other *Pseudomonas* spp. and *V. fischeri*. (Fig. 10). These inverted repeats represent binding sites of LuxR homologs, thus the presence of this putative regulatory element suggested that *pcoI* is subject to activation by a regulator of the LuxR family. The PcoI putative protein showed the highest similarity to the autoinducer synthase; whereas analysis of the PcoR putative protein revealed that contains an autoinducer-binding

domain near its amino terminus and a DNA-binding domain with a helix–turn–helix motif characteristic of the LuxR family near its carboxyl terminus.

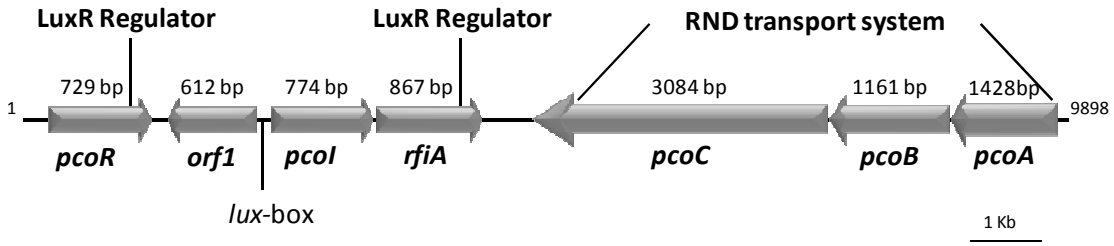


Fig. 11 - Gene map of a 9.8-kb DNA region of *Pseudomonas corrugata* CFBP 5454. The *rfiA* gene and *pcoABC* operon are localized downstream of the *pcoI/R* quorum-sensing system. *rfiA,* oriented in the same direction as *pcoI,* encodes for an LuxR transcriptional activator protein. Genes *pcoA, pcoB,* and *pcoC* form the *pcoABC* operon, which is divergently oriented, and encode for a resistance nodulation-cell-division transporter system.

Knock out mutants of *pcoI* and *pcoR* genes were generated by using the Tn-5 transposon insertion method. The *P. corrugata* GL1 (*pcoI*::Tn5) mutant no longer produced AHLs suggesting that a single QS system was present in the *P. corrugata* CFBP 5454 strain, with *pcoI* as the unique AHL-synthase. On the contrary the *P. corrugata* GL2 (*pcoR*::Tn5) mutant retained the ability to synthesize AHLs but displaying a remarkably less signal compared to the wild-type strain. Licciardello and associates also investigated on the role of the AHL-QS system in the expression of a variety of phenotypic traits. In particular, the inactivation of *pcoI* did not affect pathogenicity when inoculated in tomato, and HR in non host plant, whereas *pcoR* mutant determined reduced tomato pith necrosis symptoms, although the population sizes remained similar to that of the wild-type strain. In order to explain the different behavior, the authors hypothesized the possible involvement of a negative regulation by PcoR, or a presence of AHL mimic compounds in the plants which complement the *pcoI* mutant. Moreover the AHL QS is often integrated in other global regulatory networks, that could influence the mutants behavior.

Other difference between the two mutants were also detected (i.e: colony morphology, swimming and swarming motility, antimicrobial activity), but actually, the reason for this different phenotypic behavior between *pcoR* and *pcoI* mutants remained unknown (Licciardello *et al.*, 2007).

In further studies, in order to characterize the DNA region linked to the AHL-QS locus, Licciardello *et al.* (2009) identified located at the right border of the *pcoI* gene, a regulatory gene *rfiA.* The putative RfiA protein showed high homology with the SalA and SyrF regulators in *P. syringae* pv. *syringae* B301D (Lu *et al.*, 2002) and with the RfiA protein of *P. corrugata* 2140R, which is predicted to encode a transcriptional activator involved in the biosynthesis of antimicrobial compounds, although this gene has only been annotated as a possible regulator of fungal inhibition without experimental evidence. Sequence analysis also showed that RfiA contains a DNA-binding domain with a helix-turn-helix (HTH) motif characteristic of the LuxR family near its carboxy-terminus, consisting of four helix bundles in which the central helices form the HTH motif (Fuqua and Greenberg 2002). However, unlike the previous described LuxR homologous, PcoR (Licciardello *et al.*, 2007), RfiA does not contain the five highly conserved amino acids (Trp59, Tyr69, Asp79, Pro80, and Gly121) in the *N*-terminus characteristic of the autoinducer-binding subfamily of the QS LuxR-family proteins (Fuqua *et al.*, 1996, Finney *et al.*, 2002). Moreover, RfiA lacks the "acid pocket" characteristic of members of the response regulator subfamily domains (Parkinson and Kofoid 1992). The authors showed that in *P. corrugata*, the newly characterized *rfiA* forms an operon with the *pcoI* AHL-synthase gene and it was the first report of a *luxI* homolog cotranscribed with a regulatory gene. As consequence the transposon inserted in the *pcoI* gene (Licciardello *et al.*, 2007) has a polar effect on the cotranscribed *rfiA* gene, therefore the GL1 is a double *pcoI/rfiA* mutant.

The RfiA homologs SalA and SyrF LuxR proteins, are localized in the genomic island and regulate the syringomycin and syringopeptins CLP production (*syr-syp*) (Wang *et al.* 2006). However, in *P. syringae* pv. *syringae*, the production of CLP is not regulated by QS but is directly controlled by a complex regulatory cascade which involves the two-component sensor/regulator GacS/GacA and both transcriptional activators (Wang *et al.* 2006). Thus in order to investigate on the role of this novel transcriptional regulator in *P. corrugata* virulence and antimicrobial activity, Licciardello and collaborators created a genomic knock out mutant of *rfiA* (GLRFIA). The phenotype analysis of the GL1 (*pcoI/rfiA*-), GL2 (*pcoR*-), and GLRFIA (*rfiA*-) mutant strains revealed that they were impaired in CLP secretion because their culture filtrates were unable to inhibit the growth of the two CLP indicator microorganisms *R. pilimanae* and *B. megaterium*. The pivotal importance of RfiA in the development of the disease symptoms in tomato plants was demonstrated by the reduction of lesions and the absence of necrosis within the stem pith tissues inoculated with the *rfiA* mutant compared

with those inoculated with the WT strain CFBP 5454. Thus, these results together with those one obtained in the previous study the GL1 mutant (the double *pcoI/rfiA* mutant) was as virulent *in planta* as the WT (Licciardello *et al.*,2007), allowed to conclude that in *P. corrugata* there are two conditions in which the bacterium is virulent toward tomato: presence of PcoR in the absence of AHL or with RfiA. Therefore, in the presence of AHL, PcoR activates gene expression of virulence factors via RfiA (Licciardello *et al.*, 2009).

In conclusion the authors proposed a working model of how the PcoI/PcoR/RfiA system could be involved in virulence, in which either QS regulates the production of RfiA or PcoR regulates virulence independently of AHL (Fig. 12). Therefore, it is hypothesized that PcoR acts directly or indirectly in the absence of AHL as an activator or repressor of different virulence-associated genes.

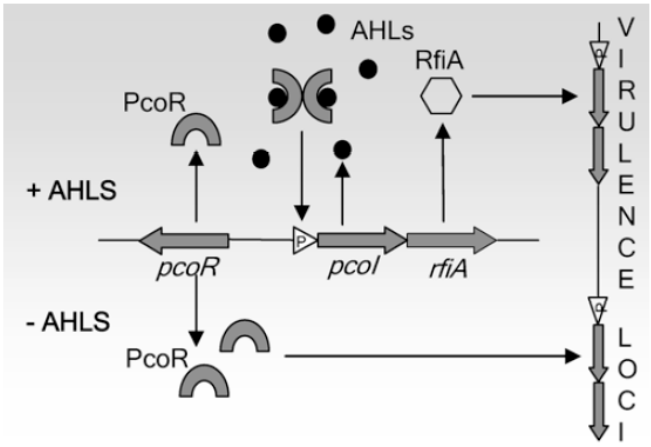


Fig. 12 - Working model for acyl-homoserine lactone (AHL) quorum-sensing (QS) and RfiA involvement in *Pseudomonas corrugata* virulence. In the presence of AHL QS, virulence is regulated via RfiA. In absence of AHL, PcoR does not activate transcription of the *pcoI/rfiA* locus and virulence is regulated by PcoR (Licciardello *et al.*, 2009).

Downstream of RfiA three ORFs transcribed in the opposite direction, which was designated *pcoA, pcoB and pcoC. pcoABC* constitute an operon and encodes three components of a tripartite resistance nodulation-cell-division (RND) transporter system were described (Licciardello *et al.*, 2009).

It consist of an outer membrane protein (PcoA), a periplasmic membrane fusion protein (PcoB), and a cytoplasmic RND transporter (PcoC) (Saier and Paulsen 2001). The predicted

PcoABC showed high homology to the PseABC efflux system described in *P. syringae* pv. *syringae* B301D, which is localized in the *syr-syp* genomic island and is involved in the secretion of syringomicin and syringopeptin CLPs (Kang and Gross 2005).

Investigations on the transporter system mutant strain GLPCOA (*pcoA*-) showed that the inactivation led only to the partial loss of antimicrobial activity against *R. pilimanae* and *B. megaterium* compared with those of the parental strain CFBP 5454. Moreover it was observed that the *pcoABC* transport system was not pivotal for virulence because GLPCOA retained its ability to be pathogenic. The *P. corrugata* PcoABC and *P. syringae* pv. *syringae*, PseABC RND transport systems share considerable homology, in fact it was demonstrated that both systems are involved in CLP secretion and that both *pcoABC* and *pseABC* mutants are as virulent as their WT strains (Kang and Gross 2005; Licciardello *et al*., 2009). In *P. syringae* pv. *syringae*, two additional ABC transporters, encoded by *syrD* and *sypD*, are also involved in the secretion of syringomycin and syringopeptins (Quigley *et al*. 1993), thus Licciardello *et al*., suggested that since the PcoABC efflux system had a role in CLP secretion but it was not essential because, most probably, *P. corrugata* possesses other efflux pumps able to transport CLPs.

The authors also demonstrated that the *pcoABC* operon is under positive regulation by RfiA and, indirectly, by the PcoI/R system. Therefore, the regulation of the *P. corrugata pcoABC* operon occurs according to a hierarchical model. In late exponential growth, the bacterium activates AHL production, which results in the formation of the AHL-PcoR complex that most probably binds the *lux*-box element in the *pcoI* promoter and activates AHL-synthase expression as well as *rfiA* transcription. RfiA, in turn, activates the transcription of the *pcoABC* operon either directly or indirectly (Licciardello *et al*., 2009) (Fig.13).

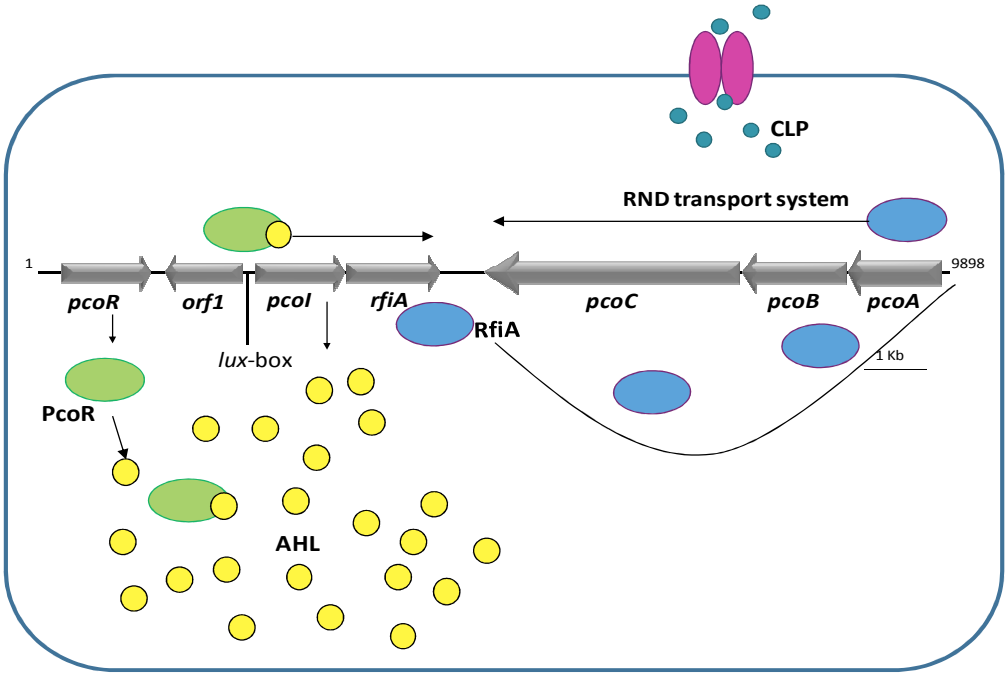


Fig. 13 - Schematic model of the AHL-QS system in *P. corrugata*. In conditions of high cell density, the complex AHL-PCOR activates the expression of *pcoI* and *rfiA,* which then activates the system type RND, *pcoABC*. In these conditions, the cell produces high concentrations of CLP which are in part transported outside the cell by the PcoABC complex.

2 . Aim of the thesis

Most plants, both economic and wild, have innate immunity or resistance to many bacteria nevertheless plant pathogenic bacteria cause many serious diseases of plants throughout the world. Because these phytopathogens have evolved either to interact with eukaryotes, but also to cope with the environment and surrounding microflora they generally exhibit a strong adaptivity, a versatile metabolism, and ingenious mechanisms of actions. This is achieved thanks to an arsenal of molecules which in turn are produced to get nourishment and reproduce, or to survive under adverse conditions.

This wide and heterogeneous array of biomolecules has been demonstrated could be further exploited for plant protection or be useful for specific biotechnological applications. Vidaver and Lambrecht (2004) for the first time pointed out the emerging evidence that a number of plant pathogens or their relatives have been widely used in agriculture and food production. They focused on non-conventional use of plant pathogenic bacteria such as the thickening agent, xanthan gum, an extra-cellular polysaccharide derived from the plant pathogen *Xanthomonas campestris* pv. *campestris* found in an enormous variety of products; the transformation or genetic engineering of plants carried out by disarmed vectors (plasmids) of *Agrobacterium tumefaciens*;; the ice-minus derivative from a nonpathogenic *Pseudomonas syringae* (the gene for ice formation was eliminated) that prevents frost damage when applied to plants. A more recent review by Tarkowski and Vereecke (2013) focus on the positive side of the top 10 plant pathogenic bacteria in molecular plant pathology (according to Mansfield et al., 2012) and some additional examples. They browsed through the full collection of published patent applications from over 90 countries using a quick search in Espacenet (*www.epo.org/espacenet*), and elaborated a list of opportunities for these pathogens[1].

Among the secondary metabolites a relevant interest is focused on lipopeptides that are compounds that are formed by cyclic (CLPs) or short linear peptides (LPs) linked with a lipid tail or other lipophilic molecules. Together with *Bacillus*, *Pseudomonas* are prominent producers of lipopeptides with a range of different natural biological activities (Raaijmakers *et al.*, 2010): antagonism of microbial competitors, protection against predators, facilitation of surface motility, biofilm formation, contribution to virulence of plant pathogens, triggering of the defense response in plants. The properties of lipopeptides may lead to applications in

diverse industrial fields including the pharmaceutical industry as conventional antibiotics; the cosmetic industry for dermatological product development due to surfactant and anti-wrinkle properties; in food production acting as emulsifiers in various foodstuffs; and also in the field of biotechnology as biosurfactants (Raaijmakers *et al.*, 2010; Mandal *et al.*, 2013).

As shown in the general introduction (cfr. 1.3.2.; 1.3.3.) *Pseudomonas corrugata* and *P. mediterranea* are examples of bacterial species described as phytopathogens that have attracted increasing interest as bioresource (Catara, 2007). In the first instance the interest focused on the use of these bacteria in applications related to agriculture both PGPR and biological control agents of plant pathogens. Subsequent studies have highlighted their potential unconventional uses as producers of the biodegradable, biocompatible biopolymers polyhydroxyalkanoates (mcl PHA).

For several years, researchers at the University of Catania and the Science and Technology Park of Sicily have been investigating the biomolecules produced by the two bacterial species in the context of several research projects between plant pathology and applied biotechnology. The molecular basis of production and the mechanisms of the interaction with plants and microorganisms, as well the potential of applications, and industrial applications have been explored[2].

[1]Patent applications on positive uses of the top 10 bacterial plant pathogens in molecular biology (according to Mansfield *et al.*, 2012) and additional phytopathogens selected based on personal expertise and interest; data obtained from www.epo.org/espacenet.

	1[a]	2[a]	3[a]	4[a]	5[a]	6[a]	7[a]	8[a]	9[a]	10[a]	11[a]	12[a]	13[a]
Total number of patents	114	62	453	82	211	15	52	12	3	13	160	14	22
Number of patents on:													
Bacterial metabolite production [b]	18			4	92		2	1			2	4	1
Biocontrol	11	1									1	2	
Bioremediation	3		6										
Protein/enzyme production	2		17	6	8				2	1		2	1
Bioconversion	3		16	1	13						1	4	
Ice nucleation	12				8								
Improvement of plant development	4		5		2		4				1	1	
Improvement of biotic/abiotic stress resistance	9		1	1	5	1	4						
Biosensor/bioassay			4								1		
Improvement of transformation methods/efficiency			198										
Generation of transgenic plants/fungi with novel properties [c]			159								44		
Use of hairy roots [d]											70		

[a] 1, *Pseudomonas syringae*; 2, *Ralstonia solanacearum*; 3, *Agrobacterium tumefaciens*; 4, *Xanthomonas oryzae*; 5, *Xanthomonas campestris*; 6, *Xanthomonas axonopodis*; 7, *Erwinia amylovora*; 8, *Xylella fastidiosa*; 9, *Dickeya*; 10, *Pectobacterium carotovorum*; 11, *Agrobacterium rhizogenes*; 12, *Rhodococcus ascians*; 13, *Streptomyces scabies* and *S. turgidiscabies*.

[b] Including secondary metabolites, polysaccharides (such as levan, alginate, xanthan,…), phytohormones, gulonic and citric acid, amides, and nucleotides.

[c] Including bioremediation, bioconversion, better resistance against biotic/abiotic stress, altered plant morphology, modified flowering time, and production of secondarymetabolites,enzymes or vaccines.

[d] Including bioremediation and production of secondary metabolites, proteins and mycorrhiza.

The biotechnology framework of the research focused in particular on two model strains of study that have both proper characteristics of the species, but at the same time showed very interesting strain dependant phenotypes. According to the results a very important role in the biology of these two bacteria is played by CLPs which are also investigated for the possibility of co-production with PHA.

The research activity of the PhD thesis aimed to provide the scientific basis on the molecular aspects of CLP production in *P. corrugata* and *P. mediterranea* and to investigate their biological role. In particular we aimed in discovering the CLPs produced by the model study strains *P. corrugata* strain CFBP 5454 and *P. mediterranea* strain CFBP 5447, to identify genes involved in CLP production and their mechanism of regulation and to shad lights on their natural role in interactions with plants and other microorganisms.

[2] Genotipi e fenotipi di interazione in batteri fitopatogeni di interesse agrario. Progetto di Ricerca di Ateneo 2008 (ex 60%), Università degli Studi di Catania.
- "Valutazione di fattori di patogenicità coinvolti nelle interazioni tra batteri fitopatogeni e pomodoro". Progetto di Ricerca di Ateneo 2007 (ex 60%), Università degli Studi di Catania.
- "Studio del Sistema di regolazione quorum sensing in *Pseudomonas corrugata*". Progetto di Ricerca di Ateneo 2006 (ex 60%), Università degli Studi di Catania.
- "Caratterizzazione molecolare di ceppi di *Pseudomonas corrugata* e di *P. mediterranea*". Progetto di Ricerca di Ateneo 2005 (ex 60%), Università degli Studi di Catania.
- "Ricerca di geni effettori in *Pseudomonas corrugata*". Programma British 2004, Partner Inglese dr.ssa Dawn Arnold Universit of West of England. Finanziato dalla Conferenza dei Rettori delle Università Italiane e dal British Council.

3. N-acyl-homoserine-lactone quorum sensing in tomato phytopathogenic *Pseudomonas* spp. is involved in the regulation of lipodepsipeptide production

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*These authors contributed equally to this work

Journal of Biotechnology 159 (2012) 274– 282

3.1. Abstract

Pseudomonas corrugata and *Pseudomonas mediterranea* are two closely related phytopathogenic bacteriaboth causal agents of tomato pith necrosis. *P. corrugata* produces phytotoxic and antimicrobial cationic lipodepsipeptides (LDPs) which are thought to act as major virulence factors. Previous studies have demonstrated that *P. corrugata* CFBP 5454 has an N-acyl homoserine lactone (AHL) quorum sensing (QS) system PcoI/PcoR and that LDP production occurs at high population densities. No molecular studies on virulence have thus far been reported for *P. mediterranea*. In this study, we show that *P. mediterranea* also produces LDPs as well as possessing an AHL-dependent QS system, designated PmeI/PmeR, which is highly homologous to the PcoI/R system of *P. corrugata* producing and responding to C6-AHL. Downstream of *pmeI*, a partial DNA sequence revealed the presence of a homolog of the *rfiA* gene of *P. corrugata* which encodes a transcriptional regulator involved in bacterial virulence. Pathogenicity tests and MALDI-TOF spectra of wild-type strains of both bacterial species and their respective QSs and *rfiA* derivative mutants revealed that, in the absence of LDPs, the strains induce very weak symptoms indicating that LDPs may act as major virulence factors. Mutational analysis of both QS systems suggests that their mode of action is in places different.

Keywords: *Pseudomonas mediterranea*, *Pseudomonas corrugata*, Quorum sensing, Lipodepsipeptides, Virulence, Tomato.

3.2. Introduction

Pseudomonas corrugata (Scarlett *et al.*, 1978) emend Sutra 1997 and *P. mediterranea* Catara *et al.*, 2002 are phytopathogenic bacteria both described as causal agents of tomato pith necrosis. Based on a number of studies evaluating taxonomy and population variability, two closely related, but distinct, taxa were outlined within the *P. corrugata* species: the taxon containing the type strain retained the name *P. corrugata,* while the second taxon was reclassified as *P. mediterranea* (Catara *et al.*, 2002; Sutra *et al.*, 1997). *P. corrugata* and *P. mediterranea* have been reported worldwide (Alippi and Lopez, 2010; Catara, 2007; Pekhtereva *et al.*, 2009; Rodrigues *et al.*, 2010) as having the same general characteristics, yet they can be clearly distinguished phenotypically and genotypically (Catara *et al.*, 2002).

P. corrugata produces phytotoxic and antimicrobial cationic lipodepsipeptides (LDPs) which are thought to be major virulence factors. Corpeptin A and Corpeptin B, two LDP isoforms of 22-amino-acid residues, were identified in the culture fluid of the *P. corrugata* type strain. They are toxic when infiltrated into tobacco leaves and antimicrobial against *Bacillus megaterium* (Emanuele *et al.*, 1998). Moreover, some of the strains produce Cormycin A, an LDP belonging to the nonapeptide lactone family acylated by a long chain of 3-hydroxy fatty acid (lipodepsinonapeptides) which produces strong *in vitro* inhibition against *B. megaterium* and *Rhodotorula pilimanae,* and exhibits phytotoxic activity (Scaloni *et al.*, 2004).

Recently, we identified an *N*-acyl homoserine lactone (AHL) quorum sensing (QS) system in *P. corrugata* strain CFBP 5454. As in other Gram-negative bacteria, the production of AHL signal molecules (mainly hexanoyl-homoserine lactone, C_6-HSL), occurs in a cell-density-dependent fashion and requires the expression of the AHL synthase gene, *pcoI,* and the *pcoR* regulator gene (Licciardello *et al.*, 2007; 2009). Our previous work also identified the protein RfiA in *P. corrugata*, an important and novel transcriptional regulator, directly linked to QS by co-transcription with *pcoI* (Licciardello *et al.*, 2009). RfiA and PcoR are required for full virulence in tomato. Mutation of either *pcoR* or *rfiA* drastically reduces virulence in tomato, whereas interestingly inoculation of tomato plants with the *pcoI* mutant leads to infections which are indistinguishable to those caused by the parent strain suggesting that in the absence of AHL and RfiA (via co-trascription),

PcoR can induce virulence gene expression via another as yet unknown mechanism (Licciardello *et al.*, 2009).

AHL QS allows bacterial populations to coordinate the expression of some traits in a cell-density-dependent manner and relies on the production of and response to signal molecules (Fuqua and Greenberg 2002). *N*-acyl homoserine lactones (AHL) are most commonly used as QS signal molecules in Gram-negative bacteria (Fuqua and Greenberg 2002; Juhas *et al.*, 2005; Venturi 2006). In plant-pathogenic bacteria, expression of virulence factors is often dependent on AHL QS, for example, as in conjugation, cell-wall-degrading enzymes and extracellular polysaccharide production (Barnard and Salmond 2007; von Bodman *et al.*, 2003).

Regulation *via* AHL QS of phytotoxin production has been demonstrated in the rice pathogen *Burkholderia glumae* where the biosynthesis and export of toxoflavin requires the ToxJ regulator, the expression of which is regulated by the TofI/R QS system (Kim *et al.*, 2004). QS involvement in *P. corrugata* LDP production and/or secretion has been postulated since time-course monitoring of LDP production in the CFBP 5454 strain showed that LDPs are produced at high population densities in parallel to AHL production. Moreover, the antimicrobial activities of QS and *rfiA* mutants are altered and their virulence is affected (Licciardello *et al.*, 2009).

No information is available on *P. mediterranea*/tomato molecular interaction, nor any differences with the closely related taxon *P. corrugata.* Both species are able to induce undistinguishable symptoms thus it is likely that they share common virulence mechanisms. It is not known whether the two species have a common ancestor or whether they have acquired separately the ability to infect plants by horizontal gene transfer. Moreover, strains of both species have been successfully tested as biological control agents in different pathosystems (Catara, 2007). *P. corrugata*'s efficacy as a biological control agent seems to be linked to its elevated rhizosphere competence. In addition, its in vitro antimicrobial activity against a long list of microorganisms (Gram-negative and Gram-positive bacteria, Chromista, yeast, fungi) shows it is able to compete by producing substances with antimicrobial activity (Catara, 2007).

The antimicrobial and phytotoxic activity of *P. mediterranea* culture fluid (Catara V., unpublished) and the production in preliminary screenings of LDPs (Fogliano V., unpublished results) prompted us to investigate the production of AHL and a QS system in this bacterial species. In this study, we report on the identification and characterization

of AHL QS of *P. mediterranea* designated PmeI/PmeR being highly homologous to the PcoI/PcoR system of *P. corrugata* and the production of LDPs also in *P. mediterranea* and correlation between virulence and LDP production in both bacterial species.

3.3. Materials and Methods

3.3.1. Bacterial strains, culture conditions and DNA manipulations

Strains and plasmids used in this study are listed in Table 4. *P. mediterranea* and *P. corrugata* strains were routinely grown at 28°C in either nutrient agar (Oxoid, Milan, Italy) supplemented with 1% dextrose (NDA), in Luria-Bertani (LB) agar or M9 minimal medium (Sambrook *et al.*, 1989). Five AHL bacterial biosensors were used: *Chromobacterium violaceum* strain CV026, *Agrobacterium tumefaciens* NTL4 (pZLR4), *Escherichia coli* JM109 (pSB401), and MT102 (pJBA132) and *P. putida* F117 (pKRC12). *E. coli* strains DH5α (Hanahan, 1983), S17-1 (Simon *et al.*, 1983), DH5α (pRK2013) (Figursky and Helinski, 1979), and HB101::Tn5 (Magazin *et al.*, 1986) were also used. *E. coli*, *C. violaceum*, *P. putida* and *A. tumefaciens* were grown in LB broth at 37, 30 and 28°C, respectively. Antibiotics were added as required at the following final concentrations: ampicillin, 100 μg mL^{-1}, streptomycin, 100 μg mL^{-1}, tetracycline, 15 μg mL^{-1} (*E. coli*), or 40 μg mL^{-1} (*Pseudomonas*); gentamycin, 10 μg mL^{-1} (*E. coli*) or 30 μg mL^{-1} (*Pseudomonas* and *Agrobacterium*); kanamycin, 50 μg mL^{-1} (*E. coli* and *C. violaceum*) or 100 μg mL^{-1} (*Pseudomonas*); chloramphenicol 25 μg mL^{-1} (*E. coli*) or 250 μg mL^{-1} (*Pseudomonas*).

DNA manipulations, including digestions with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments, ligation with T4 ligase, DNA hybridization, radioactive labeling by random priming, and *E. coli* transformation were performed as described by Sambrook *et al.*, 1989. Southern hybridizations were performed by using N+Hybond membranes (Amersham Biosciences); plasmids were purified by the alkaline lysis method (Birnboim, 1983); total DNA from *Pseudomonas* spp. was isolated by Sarkosyl-pronase lysis as described previously (Better *et al.*, 1983). Triparental matings from *E. coli* to *P. mediterranea* CFBP 5447 were carried out with the helper strain *E. coli* DH5α (pRK2013) (Figurski and Helinski, 1979), whereas plasmids were introduced into *P.*

mediterranea by biparental conjugation using *E. coli* S17-1 as donor, incubated for 22 h at 30°C.

3.3.2. Cloning of Quorum Sensing genes of *P. mediterranea*

A cosmid library of *P. mediterranea* strain CFBP 5447 harboured in *E. coli* (Bella *et al.*, 2007) was transferred by triparental conjugation to *C. violaceum* CV026 (Swift *et al.*, 1993). One cosmid, pLC13.44, could restore purple pigmentation in *C. violaceum* CV026. Plasmid pM1.9, an EcoRV subclone of pLC13.44 still able to induce violacein accumulation in CV026, was sequenced by primer walking on both DNA strands. A consensus nucleic acid sequence was prepared using Bioedit (version 5.0.9). ORF finder software at http://www.ncbi.nlm.nih.gov/gorf/gorf.html was used to determine open reading frames (ORFs) and to conduct sequence similarity searches using the BLASTP software (2.2.6) from the National Center for Biotechnology Information website. All DNA sequencing was performed either at the CRIBI centre (University of Padova, Italy) or at Macrogen. The entire sequence of the *pmeI/R* locus, was deposited in the GenBank/EMBL/DDBJ database under accession number **HQ913629**.

3.3.3. Construction of *P. mediterranea pmeR*, *pmeI* and *rfiA* knock-out mutants

Different genomic mutants were created as follows. The central parts of *pmeI* , *pmeR* and *rfiA* were amplified by PCR as 326-bp , 267-bp and 244 bp fragments, respectively, using primers PmeIKn-fw and PmeIKn-rew, PmeRKn-fw and PmeRKn-rew, RfiAKn-fw and RfiAKn-rew (Table 4) and cloned as a *Bam*HI/*Xho*I fragment in the corresponding site in pKNOCK-Km, generating pKMPmeI , pKMPmeR and pKMRfiA. These latter plasmids were then used as suicide delivery system in order to create *pmeI*, *pmeR* and *rfiA* knockout mutants through homologous recombination in strain CFBP 5447, as described previously (Alexeyev, 1999), generating PSMEI, PSMER and PSRFIA respectively. The fidelity of the marker exchange events was confirmed by Southern analysis (data not shown).

Table 4 - Bacterial strains, plasmids and primers used in this study

Strains	Genotype and /or relevant charactestics	Reference or source
P. mediterranea		
CFBP 5447	Wild type, source of *pmeR*, *pmeI* and *rfiA*	CFBP[a]
PSMER	*pmeR*:: pKnock, Km^r	This study
PSMEI	*pmeI*:: pKnock. Km^r	This study
PSRFIA	*rfiA*:: pKnock, Km^r	This study
PSMERC	PSMER mutant complemented with cosmid pLC13.44	This study
PSMEIC	PSMEI mutant complemented with cosmid pLC13.44	This study
PSRFIAC	PSRFIA mutant complemented with pBBR-RfiA	This study
PSMEI(pMPmeI)	PSMEI mutant containing the pMP190 promoter probe vector carrying the *pmeI* promoter region, Cam^r,Km^r	This study
P. corrugata		
CFBP 5454	Wild type, source of *pcoI* , *pcoR* and *rfiA*	CFBP[a]
GL1	*pcoI*362::Tn5, Km^r	Licciardello *et al*. (2007)
GL2	*pcoR*76::Tn5, Km^r	Licciardello *et al*. (2007)
GLRFIA	*rfiA::* pKnock, Km^r	Licciardello *et al*. (2009)
E. coli		
pLC13.44	pLAFR3 containing *P. mediterranea* CFBP 5447 DNA, Tc^r	Bella *et al*. (2007)[b]
DH5α	F2 f80d*laczZDM15* D(*lacZYA-argF*)*U169 endA1 recA1 hsdR17 deoR gyrA96 thi-1 relA1 supE44*	Sambrook *et al*. (1989)
S17-1	*recA pro hsdR RP4-2-Tc::Mu-Km::Tn7*, Tmp^r, Spc^r, Str^r	Simon *et al*. (1983)
JM109 (pSB401)	Tet^r, *luxR luxCDABE*	Winson *et al*.(1998)
MT102 (pJBA132)	pME6031-*luxR-PluxI*-RBSII-*gfp* (ASV)-T0-T1, Tc^r	Andersen *et al*. (2001)
A.tumefaciens		
NTL4(pZLR4)	*A. tumefaciens* NT1 derivative carrying a *traG: : lacZ* reporter	Luo *et al*. (2001)
C.violaceum		
CV026	ATCC 31532 derivative*, cviI*::Tn5*xylE* Km^r Sm^r	McClean *et al*.(1997)
P.putida		
F117(pKRC12)	Gm^r*;* pBBR1MCS-5 carrying P_{lasB} – *gfp*(ASV) P_{lac} - *lasR*	Riedel *et al*. (2001)
Plasmids		
pGEM-T	Cloning vector, Amp^r	Promega
pMP190	Promoter probe vector, low-copy-number *lacZ* fusion vector InP Cam^r, Str^r	Spaink *et al*. (1987)
pKNOCK-Km^r	Mobilizable suicide vector, Km^r	Alexeyev, (1999)
pRK2013	Km^{r}100, Tra$^+$ Mob$^+$ ColE1 replicon	Figurski and Helinski, (1979)
pKMPmeI	pKNOCK containing an internal fragment of *P. mediterranea* CFBP 5447 *pmeI* gene	This study
pKMPmeR	pKNOCK containing an internal fragment of *P. mediterranea* CFBP 5447 *pmeR* gene	This study
pKMRfiA	pKNOCK containing an internal fragment of *P.mediterranea* CFBP 5447 *rfiA* gene	This study
pMPmeI	pMP190 promoter probe vector carrying the *pmeI* promoter region, Cam^r	This study
pMPmeR	pMP190 promoter probe vector carrying the *pmeR* promoter region, Cam^r	This study
pM1.9	pMOSBlue carrying a 4.1-Kb *EcoR*V frangment containing partial pmeI/R locus, Amp^r	This study
pBBR-RfiA	pBBR1MCS-5 containing the full length *P.corrugata* CFBP 5454	Licciardello *et al*. (2009)

Oligonucleotides	Sequence	
PmeRKn-fw	5'-AGGATCCGCAAGAACTCACTC-3'	This study
PmeKn-rew	5'- ACTCGAGGGTTTTGATGATGAACAG-3'	This study
PmeIKn-fw	5'- AGGATCCCCTCCAACAAACCGACT-3'	This study
PmeIKn-rew	5'-ACTCGAGATGATGGTCTCGCCCTTTG-3'	This study
RfiaKn-fw	5'-ACTCGAGGCTGTCCGTTTGTT-3'	This study
RfiaKn-rew	5'-AGGATCCGAAATACCTGTCGA-3'	This study
PrpmeR-fw	5'-AGT GACTGTGCGCAGAGTCGGAC-3'	This study
PrpmeR-rew	5'-AGGATCCTCTTCGCTCACGCCTG-3'	This study
PrpmeI-fw	5'-ACTCGAGTTTGTCGGCCCTGGAAC-3'	This study
PrpmeI-rew	5'-AAGATCTGTTGCAGCGTTTGCGGT-3'	This study

[a]CFBP, Collection Francaise de Bacteries Phytopathogenes, Angers, France.

3.3.4. Reporter gene fusion assay

Transcriptional fusion plasmids for *pmeR* and *pmeI* promoter regions based on the pMP190 promoter probe vector were constructed as follows. The 519-bp and 736-bp fragments containing the *pmeR* and *pmeI* promoter region, respectively, were amplified by PCR by using genomic DNA of *P. mediterranea* CFBP 5447 as the template, and oligonucleotides PrpmeR-fw and PrpmeR-rew, PrpmeI-fw and PrpmeI-rew, respectively (Table 4). The DNA fragments were then cloned into pGEM-T (Promega), removed as *Sal*I/*Bam*HI or *Xho*I/*Bgl*II and cloned in pMP190 yielding pMPmeR and pMPmeI, respectively. β–Galactosidase activities were determined during growth in LB medium essentially as described by Miller (1972), with the modification of Stachel and associates (1985). All experiments were performed in triplicates and the mean value is given. β–Galactosidase activities were determined after a 50-ml LB medium culture started with an initial inoculum of 1.7×10^8 CFU.

3.3.5. Detection, isolation and quantification of AHLs

To survey AHLs production by *P. mediterranea* a T-streak plate assay was performed as described by Piper *et al.*, (1993) on LB agar plates. *P. mediterranea* CFBP 5447, PSMEI and PSMER mutant derivatives AHLs were further characterized by TLC after AHL-extraction from spent supernatants essentially as described previously (McClean *et al.*, 1997; Shaw *et al.*, 1997). Synthetic AHLs (including C6-3-oxo-AHL, C8-3-oxo-AHL, C10-3-oxo-AHL, C4-AHL, C6-AHL, C7-AHL and C8-AHL) were used as standard molecules. *C. violaceum* CV026, *E. coli* JM109 (pSB401) and *A. tumefaciens* NTL4 (pZLR4) were used as

indicator strains. Quantification of AHLs in the extracts was indirectly determined using the *P. mediterranea* PSMEI(pMPmeI) as sensor. Overnight cultures of PSMEI(pMPmeI) were diluted in 10 mL of LB medium to an A660 of 0.35; the AHL extracts to be quantified were then added and after 3 hours of growth β-galactosidase activity was determined (Bertani *et al.*, 2007).

3.3.6. Pathogenicity test

Pathogenicity of *P. mediterranea* CFBP 5447 and derivative mutants was confirmed on tomato plants (cv. Moneymaker) in a growth chamber with 16/8 h photoperiod and 26°C temperature essentially as described by Licciardello *et al.* (2007; 2009). Two months old tomato seedlings were pin-prick inoculated on the stem at the axil of the first true leaf (20 plants per strain) with bacterial cells from 48-hrs culture on NDA. Plants were rated for symptoms 15 days after inoculation. Experiments were performed at least twice. A host–pathogen interaction phenotype was evaluated using an empirical 0-to-5 increasing susceptibility scale based on the range of symptoms and results expressed as mean DI; a point each was added if additional symptoms were observed (i.e. hollowing of the pith, presence of brown discoloration of the xylem beyond the TPN-affected areas) (Licciardello *et al.*, 2009).

3.3.7. *P. mediterranea* and *P. corrugata* lipodepsipeptide production

P. mediterranea CFBP 5447, *P. corrugata* CFBP 5454 and respective mutant derivatives were evaluated for LDP production in culture filtrates by MALDI-TOF mass spectrometry analysis. Culture filtrates were prepared in IMM (Improved Minimal Medium) as previously described (Licciardello *et al.*, 2009). The samples were dissolved in a solution of 49.9% water, 0.1% Trifluoroacetic Acid and 50% CH_3CN before to be analyzed through the mass spectrometer. MALDI-TOF mass spectrometry experiments were carried out on a Voyager DEPRO mass spectrometer (PerSeptive Biosystems, Framingham, MA) equipped with an N2 laser (337 nm, 3 ns pulse width) operating both in the linear and reflector positive ion modes using the Delayed Extraction. Samples were co-crystallised with 3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enoic acid (sinapic acid) as the matrix, which was prepared by dissolving 10 mg of sinapic acid in 1 mL of aqueous 50% (v/v)

acetonitrile containing 0.1% (v/v) Trifluoroacetic Acid. Raw data were elaborated using the software program Data Explorer version 4.0 (Applied Biosystems).

To test all strains for biosurfactant activities, culture filtrates 10× concentrated were used. After the addition of 5% methylene blue, 10 µl were pipetted as a droplet onto Parafilm and the spreading of the droplet was observed (Vallet – Gely *et al.*, 2009).

3.4. Results and Discussion

3.4.1 *P. mediterranea* possesses an AHL-QS system highly homologous to that of *P. corrugata*.

P. mediterranea type strain CFBP 5447, just like *P. corrugata*, was found to possess one AHL-dependent QS system, designated PmeI/R (Fig. 14). Ten *P. mediterranea* strains, isolated from various parts of the world, were initially tested for their ability to produce AHLs by T-streaking them with biosensors *C. violaceum* CV026, JM109 (pJBA132) and *P. putida* F117 (pRKC12); a positive response was obtained for all 10 strains with CV026 and JM109 (pJBA132) indicating the probable presence of short- medium-chain length AHLs (data not shown). The TLC of the ethyl acetate extracts of *P. mediterranea* strain CFBP 5447 culture supernatant showed that it mainly produces one signal molecule, tentatively identified as C_6-AHL (Fig. 15A). Two additional spots could be seen when the TLC plate was overlaid with the *Agrobacterium* NTL4 biosensor; the retention factor (Rf) of these spots potentially corresponded to that of C_8-AHL and C_6-3-oxo-AHL standards (data not shown).

To identify the gene(s) responsible for AHL synthesis we conjugated *en masse* a genomic library of *P. mediterranea* CFBP 5447 into the AHL biosensor *C. violaceum* CV026. This procedure identified the AHL QS locus within a 4.099-bp *Eco*RV fragment. The DNA sequence comprises two incomplete open reading frames (ORF) from positions 1 to 348 and 3598 to 4099, and three complete ORFs (Fig. 14).

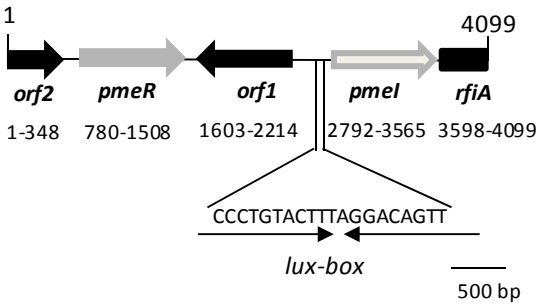

Fig. 14 - Physical map of the 4,099 bp *Eco*RV fragment containing the AHL quorum sensing system of *P. mediterranea* CFBP 5447. Gene position and orientation is highly similar to the homologous region described in *P. corrugata* CFBP 5454. The *pmeR* and *pmeI* genes are shown as well as the putative 20-bp *lux-box* sequence with dyad symmetry, as indicated by the convergent arrows, located upstream of the putative transcription start site of *pmeI*. In the downstream region of *pmeI,* partial *rfiA,* similar to LuxR transcriptional regulator, is located.

The first complete ORF spans 729 bases and encoded a predicted 242-amino acid protein. Since analysis of the putative protein revealed that it contains an autoinducer-binding domain near its amino terminus (amino acid 24-170) and a DNA-binding domain with the helix-turn-helix motif characteristic of the LuxR family near its carboxyl-terminus (amino acids 179-236) it was designated as PmeR. PmeR was very similar to the PcoR transcriptional activator (**ABP88722**) described in the QS system of *P. corrugata* CFBP 5454 showing 96% of sequence identity (99% similarity). Downstream *pmeR* a second ORF encoding a putative 203-amino-acid protein was present. This ORF, named ORF1, is 93% identical (96% similarity) to a putative membrane protein identified in *P. corrugata* CFBP 5454 (**ABP88723**) and 56% identical (75% similarity) to the hypothetical protein PsyrpsF_13067 of *P. syringae* pv. *syringae* FF5 (**ZP06495076**) involved in amino acid transport and metabolism.

The third complete ORF proves to be *luxI* homolog, and was named *pmeI.* It spans 774 bases and is predicted to encode a 257-amino acid protein homologous to members of the LuxI family. The PmeI putative protein has 85% identity (91% similarity) with the *P. corrugata* 2140R PcoI protein (**AAG28558**) and 85% identity (88% similarity) with the PcoI of *P. corrugata* CFBP 5454 (**ABP88722**). In the putative promoter region of *pmeI* we identified a 20-bp imperfect palindrome, centered 67 bp upstream from the putative translational start site, which shares a high degree of similarity with *lux* box-like elements

(Fuqua *et al.*, 1994) (Fig. 14). No differences were found with the *lux* box-like element described in *P. corrugata* CFBP 5454 (Licciardello *et al.*, 2007). This putative regulatory element, which represent the binding sites for the LuxR homologue, suggests that *pmeI* is subject to activation by a regulator of the LuxR family.

PmeI and PcoI, as well as PmeR and PcoR showed the highest homologies to LuxI and LuxR family proteins of other *Pseudomonas* principally with strains of different pathovars of *P. syringae* and other oxidase negative *Pseudomonas* plant associated bacterial species other than to oxidase positive species such as *P. fluorescens* and *P. putida*. By contrast, DNA sequence analysis of rDNA genes and other reference genes in phylogenetic and taxonomic studies ascertained that *P. corrugata* and *P. mediterranea* are more similar to species within the *P. fluorescens* clade than to those in *P. syringae* (Cladera *et al.*, 2006; Mulet *et al.*, 2010). Differences between phylogeny and quorum sensing protein analysis had already been observed. In particular Lerat and Moran (2004) observed that LuxI and LuxR proteins are clearly subdivided into two different families of homologous genes. LuxI and LuxR proteins from *P. fluorescens*, *P. aeruginosa*, *P. chlororaphis* clustered in family A whereas those from *P. syringae* clustered in the B group with those of *Enterobacteriaceae,* hence presumably those of *P. corrugata* and *P. mediterranea*.

The deduced amino acid sequence of the truncated ORF located upstream of *pmeR* (ORF2) showed high sequence similarity to a putative excinuclease ABC and subunit in *P. putida* and *P. fluorescens* (>80%). The partial ORF located downstream of *pmeI* (502 bp), transcribed in the same direction and separated from it by only 32 bp, showed sequence similarities of 93% and 92% with the *rfiA* gene sequences of *P. corrugata* strains 2140R (**AF199370**) and CFBP 5454 (**EF189721**). The RfiA putative protein of *P. corrugata* CFBP 5454 contains an HTH DNA motif in the C-terminus characteristic of the LuxR family of bacterial regulatory proteins but lacks the conserved residues at the N-terminus characteristic of the autoinducer-binding subfamily of the QS LuxR-family proteins. De Bruijin and Raaijmakers (2009) observed by in silico DNA analysis that these LuxR-type regulators are positioned up and downstream of the LDP biosynthesis genes of various *Pseudomonas*. In *P. corrugata* CFBP 5454, RfiA, whose expression is directly regulated by AHL QS via *pcoI-rfiA* co-trascription, is crucial to bacterial virulence in tomato and antimicrobial activity (Licciardello *et al.*, 2009).

Even if the 4,099 bp DNA fragment and putatively coded proteins showed high nucleotide and amino acid homologies to those of *P. corrugata* CFBP 5454 (data not shown), a significant difference was observed between the promoter region of *pmeR* and *pcoR*. In fact, the promoter region of *P. mediterranea* is 35 bp longer than that of *P. corrugata*. This 35 bp sequence is located 79 bp upstream of the putative translational site of *pmeR*; over the entire sequence only 4 nucleotides (AGCT), located centrally, appear in the corresponding region of *P. corrugata.* It is currently not known whether this difference produces different regulation of *pmeR* compared to *pcoR*.

The *pmeI*, *pmeR* and *rfiA* genes were insertionally inactivated independently creating three genomic mutants of *P. mediterranea* designated PSMEI, PSMER and PSRFIA (Table 4). Both *P. mediterranea* QS mutants, PSMEI and PSMER, no longer produced AHLs as determined by TLC analysis (Fig. 15A). In order to quantify AHLs the *pmeI* promoter region was cloned into the pMP190 reporter plasmid based on *lacZ* activity yielding pMPmeI. This transcriptional fusion was transformed into the *P. mediterranea pmeI* mutant generating strain PMEI(pMPmeI) which was used as an AHL detection sensor of the parent strain and QS derivative mutant supernatants showing that AHL production was abolished in both mutants (Fig. 15B). *PmeI* promoter activity was strongly reduced both in the *pmeR* mutant as well as in the *pmeI* mutant as compared to the *pmeI* promoter activity levels which demonstrate that control of *pmeI* transcription is affected by the PmeR-AHL complex (Fig. 16A).These results showed that *P. mediterranea* possesses one AHL QS system and that *pmeI* is most likely the only AHL-synthase in the genome. Mutants were complemented for AHL production when cosmid pLC13.44 was introduced by tri-parental conjugation (data not shown), the complemented mutants were designated PSMEIC and PSMERC.

To determine whether *pmeR* and *pmeI* expression was regulated in a canonical AHL QS way, the activity of the two promoter regions was analyzed. The activity of the *pmeR* promoter (Fig. 16B) is similar in PSMEI, PSMER and the parental strain suggesting that *pmeR* was not self-regulating and that no *lux-box* like element was found in its upstream region. *PmeI* promoter activity, on the other hand, was undetecTable 4in the QS *P. mediterranea* mutant derivatives, indicating that it is under their positive regulation creating a positive-feedback loop typical of AHL QS systems (Venturi, 2006).

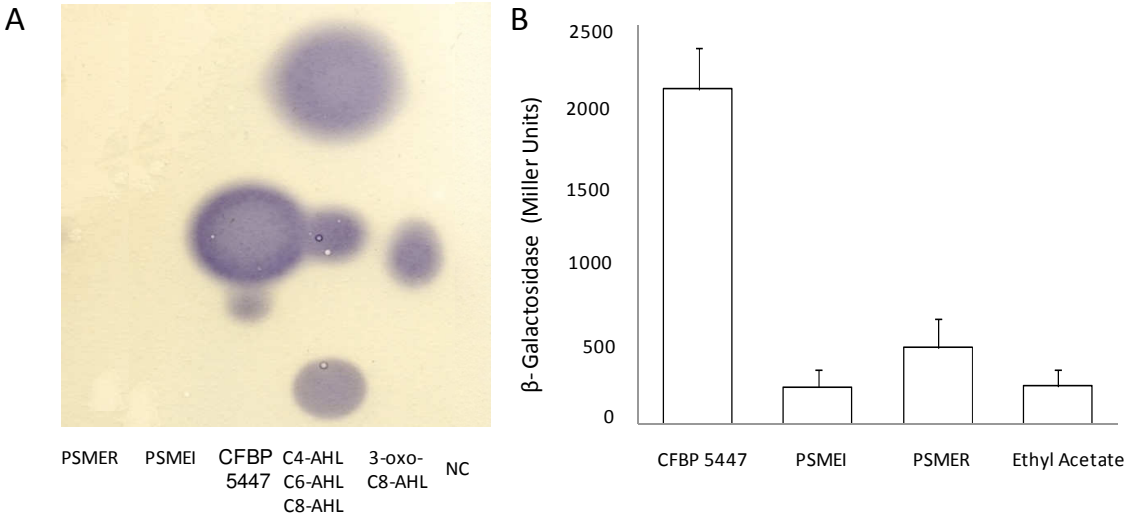

Fig. 15 - (A) TLC analysis of AHLs produced by *P. mediterranea* CFBP 5447 parent strain, PSMER (*pmeR-)* and PSMEI *(pmeI-)* mutant derivatives. Strain *C. violaceum* CV026 was overlaid on the TLC as AHL biosensor. Synthetic AHL were included as reference compounds. NC: negative control (uninoculated M9 medium). (B) AHLs were quantitatively detected after extraction from spent supernatants of *P. mediterranea* CFBP 5447 (parent strain) and the QS mutant derivatives PSMEI and PSMER by measuring *pmeI* promoter activity of the reporter strain PSMEI(pMPmeI) (cfr. 2.5 and Tab. 1). β-Galactosidase activity (Miller Units) was measured in three independent cultures for each strain. Bars represent standard deviation for the replicates.

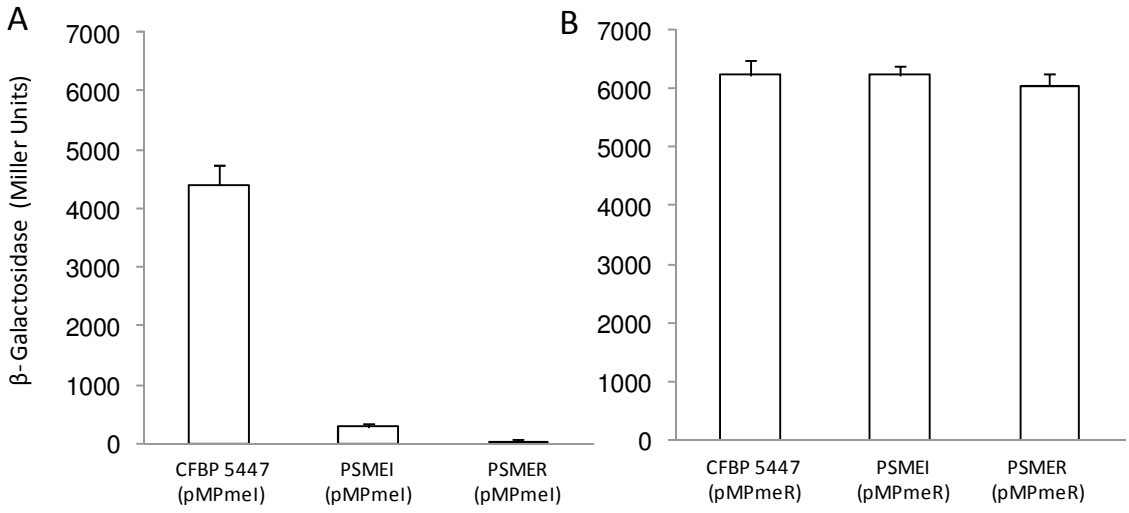

Fig. 16 - Gene promoter activities of *pmeI* (A) and *pmeR* (B) in *P. mediterranea* CFBP 5447 and quorum sensing mutant derivatives. Promoter activities were expressed in β-galactosidase activity (Miller Unit) as the plasmid constructs pMPmeI (A) and pMPmeR (B) contains the *pmeI* and *pmeR* promoters, respectively, transcriptionally fused to a promoterless *lacZ* gene. Standard deviation bars are given on the mean value of three independent experiments. *pmeI* gene expression is under positive feedback *QS* regulation, whereas *pmeR i*s not affected by QS.

3.4.2. AHL-QS system is involved in *P. mediterranea* virulence

Tomato plantlet stems were inoculated with *P. mediterranea* CFBP 5447 parental strain and PSMEI (*pmeI*-), PSMER (*pmeR*-) and PSRFIA (*rfiA*-) knock-out mutant derivatives to determine the importance of *P. mediterranea* AHL QS in bacterial virulence, which was evaluated by eye and by disease rating scores based on symptoms and used to calculate a mean disease index (DI) (Fig. 17A). In plants inoculated with the parent strain, the stem pith appeared dark brown and sometimes necrotic, hollowed with ladder-like lesions. Symptom extension could vary from 2.5 to up to 12 cm (Fig. 17B). The PSMEI and PSMER mutants caused only light brown discolorations inside the stem, never hollowed and significantly reduced in length compared to those caused by the parent strain; plants inoculated with the PSRFIA mutant showed only light brown discoloration at the inoculation site.

Complementation was performed using the QS locus of *P. mediterranea* which was able to restore the virulence ability of the mutants. Similar results were obtained when *P. mediterranea rfiA* mutant (PSRFIA) was complemented *in trans* with the pBBR-RfiA construct containing the homologue *rfiA* gene of *P.corrugata* CFBP 5454. Plants co-inoculated with the PSMEI strain and exogenous AHLs showed significant worsening of the symptoms but not to full restoration (i.e. increase of the lesion extension and necrosis). The restoration of the virulent phenotype obtained using RfiA alone *in trans* under a QS independent promoter (pBBR-RfiA) and the failure of chemical complementation of the PSMEI mutant using exogenous AHLs, suggest that the AHL-QS system of *P. mediterranea* is regulating the virulence mainly by the co-transcription of *pmeI-rfiA*. This locus therefore most probably works as in *P. corrugata* where *pcoI* and *rfiA* are part of the same transcriptional unit, and it was concluded that *rfiA* was also under the control of the *pcoI* promoter region (Licciardello *et al.*, 2009).

Interestingly, some differences with *P. corrugata* are evident. In fact, in *P. corrugata* the *pcoR* mutant showed drastically reduced virulence whereas the *pcoI* mutant, which was actually demonstrated to be a pcoI/rfiA double mutant due to the polar effect on *rfiA* of the *pcoI* mutation, retained virulence similar to that of the parent strain. (Licciardello *et al.*, 2007; 2009). *In P. corrugata,* the addition of exogenous AHL to the *pcoI/rfiA* mutant caused a drastic reduction in virulence. It was therefore suggested that in *P. corrugata* both regulators PcoR and RfiA play a role in bacterial virulence in tomato, the sole presence of PcoR in the cell being sufficient for *P. corrugata* to be pathogenic. However,

when PcoR is in the cell with AHL and no RfiA, *P. corrugata* is less virulent. In both bacterial species RfiA is crucial for bacterial virulence. Like other LuxR-type regulators, RfiA does not require AHLs to restore pathogenicity in the *pcoR* or the *pmeI* mutant *is* in the absence of AHLs.

3.4.3. *P. mediterranea* and *P. corrugata* QS mutants showed distinguishable phenotypes

In planta results prompted us to explore phenotypic differences between the two bacterial species. *P. corrugata* epithet derives from the wrinkled (i.e. corrugata) surface of bacterial colonies on nutrient agar supplemented with 1% D-glucose (NDA) when the species was first described (Scarlet *et al.*, 1978). Most of the strains of both *P. corrugata* and *P. mediterranea* showed slightly raised colonies with wrinkled surfaces and undulated margins (Catara *et al.*, 2002). Wild-type strains of *P. mediterranea* CFBP 5447, analysed in this study, and *P. corrugata* strain CFBP 5454 studied by Licciardello *et al.* (2007), showed typical colony morphology when grown on NDA. The *P. mediterranea* PSMEI (*pmeI-)* and PSMER (*pmeR*-) mutants lost their wrinkled surface and the colonies were slightly raised to umbonate, with a smooth surface and undulated margins (Fig. 17C). Interestingly, the *P. corrugata* GL2 (*pcoR*-) mutant showed slightly raised colonies, with entire margins and smooth surfaces whereas the GL1 (*pcoI*-) mutant showed colonies indistinguishable to those of the parent strain (Fig. 17C). The RfiA null mutants of both species produced colonies that showed a smooth surface, entire margins and were considerably more mucous than the WT strains (Fig. 17C).

We also compared the mutants for active surface molecules using the 'drop collapsing assay'. When no surfactants are produced, the droplets stay round, whereas with surfactants, the droplets flatten out (Vallet–Gely *et al.*, 2009). *P. mediterranea* CFBP 5447 and *P. corrugata* CFBP 5454 produce molecules which can strongly decrease the surface tension of the culture medium (Fig.4D). These molecules are not produced by the *P. mediterranea* mutants PSMEI, PSMER and PSRFIA. The *P. corrugata* mutants GL2 (*pcoR*-) and GLRFIA (*rfiA*-) do not produce bio-surfactants whereas the GL1 (*pcoI*-) mutant retained this ability as the WT strain (Fig. 17D). These results suggested that in *P. mediterranea,* bio-surfactant production is regulated by QS as also reported for *P. syringae* pv. tabaci (Taguchi *et al.*, 2006) and other phytopathogenic bacteria (D'aes *et al.*, 2010). In *P.corrugata* another more intricate network is most probably taking place.

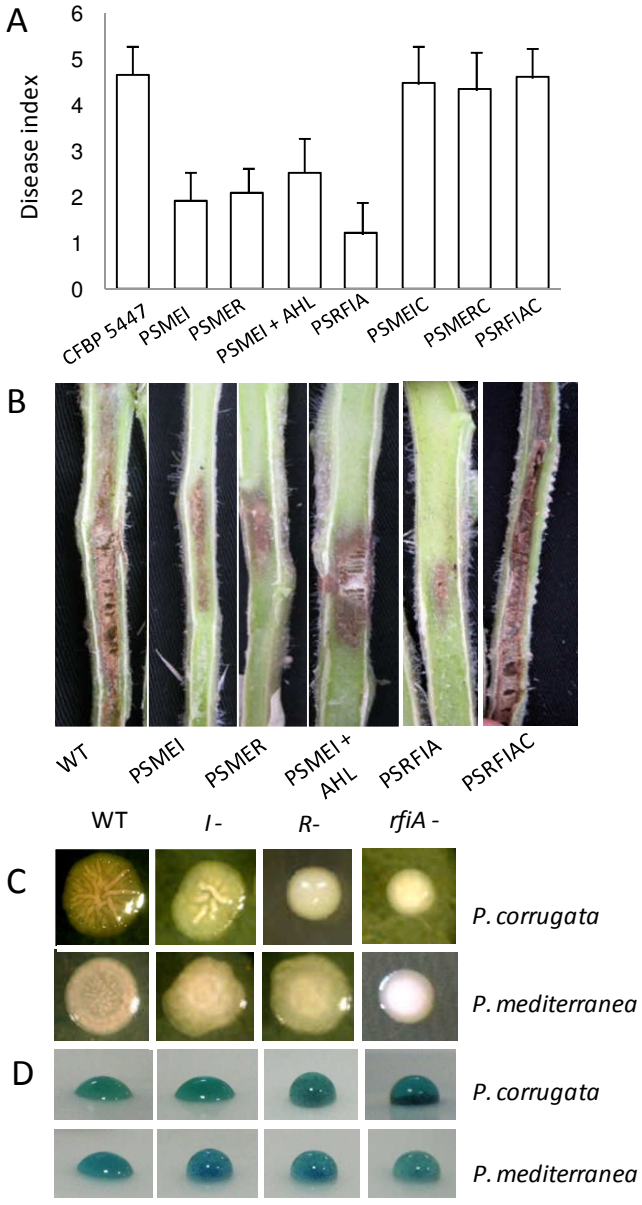

Fig. 17 - (A) Mean disease index (DI) of tomato plants inoculated with the parent strain *P. mediterranea* CFBP 5447, PSMER (*pmeR* mutant), *PSMEI (pmeI* mutant), *PSMEI* with the addition of exogenous acyl-homoserine lactones (PSMEI+AHL), PSRFIA (*rfiA* mutant) and PSMERC, PSMEIC, PSRFIAC (complemented mutants in trans). (B) Stem pith symptoms in tomato plants prick inoculated with bacterial cells of the *P. mediterranea* parent strain, PSMEI, PSMER, PSMEI+AHL, PSRFIA and PSRFIA complemented in trans with the *rfiA* gene of *P. corrugata* CFBP 5454. (C) Colony morphology of *P. corrugata* and *P. mediterranea* all strains by optical microscopy on NDA agar plates after 2 days incubation at 28°C. (D) Biosurfactant activity of the *P. corrugata* and *P. mediterranea* all strains was visualized by the drop-collapsing assay.

3.4.4. Role of QS in LDP production by *P. mediterranea* CFBP 5447 and *P. corrugata* CFBP 5454

P. corrugata produces phytotoxic LDPs (Emanuele *et al.*, 1998; Scaloni *et al.*, 2004) which are versatile metabolites produced by *Pseudomonas* spp. exhibiting potent surfactant properties (Raaijmakers *et al.*, 2006). Since, the *P. mediterranea* PSMEI (*pmeI*-), PSMER (*pmeR*-) and PSRFIA (*rfiA*-) mutants (this study) as well as the *P. corrugata* CFBP 5454, GL2 (*pcoR*-) and GLRFIA (*rfiA*-) (Licciardello *et al.*, 2007; 2009) mutants were significantly less virulent than the respective wild-type strains, and produced variable bio-surfactant activity (see above), we determined LDP production in culture filtrates of both the parental strains and respective derivative mutants by MALDI-TOF MS analysis.

The culture filtrates of *P. mediterranea* CFBP 5447 and *P. corrugata* CFBP 5454 showed specific peaks at *m/z* of 1274 and *m/z* 2095.3 and 2121.2 already observed in the *P. corrugata* strain IPVCT 10-3 (Scaloni *et al.*, 2004) (Fig. 18). Moreover, in the *P. mediterranea* culture filtrate pattern, additional peaks were observed (Fig. 18). It was observed that *P. mediterranea* produces a pattern of metabolites with masses starting at the *M*-H^+ ion at *m/z* 2066.7 up to the *M*-H^+ ion at *m/z* 2162 with a difference of 14 u that which may be due to an additional -CH_2 group in the acyl chain. So a family of LDPs similar to Corpeptin A and B but with more heterogeneity in the fatty acid moiety was produced by this strain. Moreover, we observed in the *P. mediterranea* pattern profile an additional *M*-H^+ ion at *m/z* 1366 located in the same molecular mass range of other *Pseudomonas* lipodepsinonapeptides. We hypothesized that it could be a bioactive (toxic) compound suggesting that a still putatively unidentified lipodepsipeptide is also produced. No LDPs are visible in the MALDI-TOF MS spectra of *P. mediterranea* QS mutants (i.e. flat-spectra; data not shown). *P. corrugata pcoR*- no longer produced LDPs whereas both Cormycin and Corpeptins are still produced by the *pcoI* mutant although at a reduced level compared to the parent strain (data not shown). Inactivation of *rfiA* in both species resulted in LDP deficiency as revealed by the absence of specific peaks in the MALDI-TOF MS spectra. These results support *in planta* bacterial virulence, with mutants showing no symptoms or only slight stem discolorations and unable to further produce LDPs. As a matter of fact the *P. corrugata pcoI* mutant still able to produce LDPs maintained its virulence.

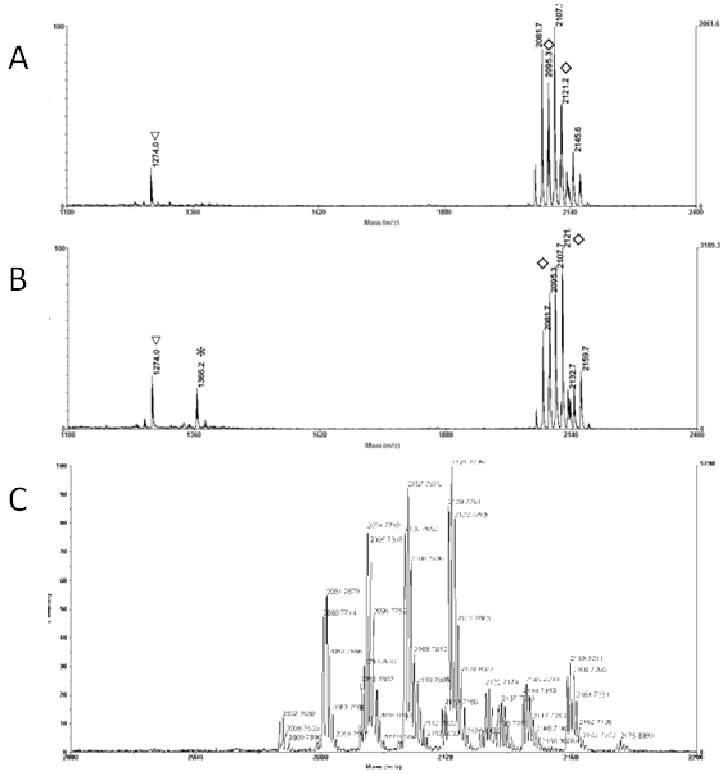

Fig.18 - (A-B) Representative MALDI-TOF mass spectra of *P. corrugata* CFBP 5454 and *P. mediterranea* CFBP 5447 cell-free extracts. The triangles label the peaks corresponding to Cormycin. The rumbles label the peaks corresponding to Corpeptins. The asterisk labels the pick arising from an additional unidentified metabolite observed in *P. mediterranea.* (C) MALDI-TOF mass spectrum in the range between 2000 m/z to 2200 m/z of *P. mediterranea* extracts. It indicates the production of a pattern metabolites having a differences of 14 u probably due to an additional –CH2 group in the acyl chain.

3.5. Conclusions

In this study we isolated and analyzed the QS systems of the plant pathogen *P. mediterranea*, a bacterial species closely related to *P. corrugata.* It was established that *P. mediterranea* harbours one system designated PmeI/R that is highly homologous to the PcoI/R system of *P. corrugata*.

P. corrugata and *P. mediterranea* QS proteins were highly homologous to the those of *P. syringae,* another phytopathogenic bacterium. Both *P. corrugata* and *P. mediterranea* are causal agent of tomato pith necrosis and here we demonstrated that

virulence on tomatoes is affected by inactivation of QS genes in both species but with some differences revealed particularly by the comparison of "I" mutants.

A LuxR regulator *rfiA* found downstream the *luxI* homologs in both species was also essential for virulence confirming the essential role in controlling virulence factor expression. MALDI-TOF analysis showed that both species in this study produce LDPs. *P. mediterranea*, alike *P. corrugata*, produces Corpeptins, and Cormycin A. A putatively unidentified bioactive compound having MW of 1366 has been also observed. *P. mediterranea* and *P. corrugata* QS and *rfiA* mutants that showed a drastic reduction of virulence when inoculated in tomato plants are also impaired in LDP production, suggesting that LDPs are pivotal for developing necrosis in tomato stem pith. The QS system of both bacterial species and the RfiA transcriptional regulators seem to have a role in LDPs production/secretion. The role of QS in LDP production was demonstrated in a number of saprophytic *Pseudomonas* but it was not clearly shown in phytopathogenic *Pseudomonas* spp. (D'aes *et al.,* 2010). In *P. syringae* the production of syringopeptins and syringomycin is not controlled by QS but by the two component GacA/GacS system through the non-QS LuxR-type transcriptional activators SalA and SyrF (Lu *et al.*, 2005). Genomic inactivation of non-QS LuxR-type regulatory genes in *P. fluorescens* SBW25 resulted in viscosin deficiency and reduced transcript levels of viscosin biosynthesis genes (de Bruijin and Raaijmakers, 2009).

The results in this study provide new, interesting insights in the behavior of two closely related bacterial species that induce the same disease. Future work will involve the identification of LDP biosynthesis gene cluster in order to investigate the role of QS and RfiA in their production.

Acknowledgements

This work was supported by the project "PolyBio-Plast" PON01 01377 funded by the Italian MIUR (Ministero dell'Istruzione, dell'Università e della Ricerca) PON 2007–2013 and a grant from the University of Catania 'Progetti di Ricerca d'Ateneo'. IB was supported by a fellowship from the Italian Cystic Fibrosis Research Foundation (Project FFC #9/2007) with the contribution of the Delegazione FFC di Belluno.

4. CrpCDE are part of the cyclic lipopeptide corpeptin biosynthetic cluster and are involved in *Pseudomonas corrugata* virulence

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4.1. Abstract

Pseudomonas corrugata CFBP 5454 produces two kind of cyclic lipopeptides (CLPs), cormycin A and corpeptins, which possess surfactant, antimicrobial and phytotoxic activity. In this study we identified a putative ABC type transport system and a non-ribosomal peptide synthetase (NRPS) involved in corpeptin production. The encoding genes were designated *crpC* and *crpDE,* respectively and constitute an operon. The genetic organization of this locus shows high similarity with other *Pseudomonas* CLP biosynthetic clusters. MALDI-TOF MS analysis revealed that transporter and synthetase genomic knock-out mutants are unable to produce corpeptins, but can still synthesize cormycin A. Moreover gene expression analysis revealed that the putative corpeptin synthetase gene *crpC* is expressed in the transporter mutant strain suggesting that corpeptins are produced by the mutant strain but not released outside the cell. Strains depleted in corpeptin production were significantly less virulent than the wild type strain when inoculated in tomato plants and their antimicrobial activity against CLP-sensitive microorganisms *Rhodotorula pilimanae* and *Bacillus megaterium* was considerably reduced. Expression studies revealed that corpeptin NRPS is best expressed at high cell density, upon reaching the quorum, and that its expression could be anticipated by addition of exogenous *N*-acyl homoserine lactone (AHL) quorum sensing signals. *CrpC* expression, as well as corpeptin production, was impaired in the *pcoR* and *rfiA* null mutants (these are *luxR*-family genes, the first being part of the AHL quorum sensing system) whereas in the *pcoI* null mutant ,(the *luxI*-family AHL synthase mutant) is still expressed at the early phases of growth, although at very reduced levels.

Keywords: *Pseudomonas*, non ribosomal peptide, ABC transporters, corpeptin, quorum sensing

4.2. Introduction

Pseudomonas corrugata Roberts and Scarlett 1981 emend. Sutra *et al.* 1997, is a ubiquitous bacterium isolated from a wide variety of sources (Catara, 2007). It was first described in the UK in the late seventies (Scarlett *et al.*, 1978) as the causal agent of tomato pith necrosis (TPN) and later associated to TPN on tomato worldwide but also occasionally on pepper, chrysanthemum and geranium (Catara 2007). The most characteristic symptom of the disease is stem pith necrosis where pith appears as necrotic and frequently disaggregated in the core. *P. corrugata* has also been isolated from non-diseased plants, mainly as an endophyte or from the rhizosphere, and has also been recovered from bulk soil (Catara 2007).

P. corrugata produces the cyclic lipopeptides (CLPs) corpeptin A and corpeptin B (CP-A and CP-B), two isoforms consisting of 22-amino-acid (AA) residues, which are toxic when inoculated into tobacco leaves and are also antimicrobial against *Bacillus megaterium* (Emanuele *et al.*, 1998). Moreover, some of the strains also produce cormycin A (CM-A), a lipodepsinonapeptide which has antimicrobial activity against *B. megaterium* but also *Rhodotorula pilimanae,* and exhibits phytotoxic activity. CM-A has higher antimicrobial and haemolytic activity than other lipodepsinonapeptides from *P. syringae* (Scaloni *et al.*, 2004). Corpeptin structure, as determined by Emanuele *et al.*, (1998), is strictly related to that of peptin-like CLPs which include fuscopeptins produced by the phytopathogen *P. fuscovaginae* (Ballio *et al.*, 1996), syringopeptins, synthesized by phytopathogenic strains of *Pseudomonas syringae* pv. *syringae* (Ballio *et al.*, 1991), tolaasin, produced by the mushroom infecting saprophytic *Pseudomonas tolaasii* (Coraiola *et al.*, 2006).

Their peptide chain range from 18 to 25 AA. A more detailed structural classification separate syringopeptins from this group based on the number of the AA residues constituting the lactone ring (Gross and Looper, 2009). Cormycin belongs to the group of the smaller nonapeptides (Scaloni *et al.*, 2004) among them the syringomicin produced by strains of *P. syringae* pv. *syringae* is the best studied (Bender *et al.*, 1999; Raaijmakers *et al.*, 2006). CLPs produced by *Pseudomonas* spp. are composed of a fatty acid tail linked to a short oligopeptide which is cyclized to form a lactone ring between two amino acids in the peptide chain (Raaijmakers *et al.*, 2006). CLPs are also called

lipodepsipeptides (LDPs) where "depsi" indicate the replacement by ester bond of the two amino acids in the cyclization reaction to form the lactone ring.

Our previous studies demonstrated that *P. corrugata* CFBP 5454 has an system-acyl-homoserine lactone quorum sensing (AHL-QS) system, PcoI/PcoR, consisting of an AHL synthase, PcoI, and a transcriptional sensor/regulator belonging to the LuxR family protein, PcoR (Licciardello *et al*., 2007). Downstream of *pcoI,* a regulator called *rfiA* is present which transcriptionally co-transcribed with *pcoI* (Licciardello *et al*., 2009). PcoR activates *pcoI* expression in the presence of exogenous AHL via a typical positive-feedback regulatory loop. As *pcoI* and *rfiA* constitute an operon, the expression of *rfiA* is directly regulated by the PcoR-AHL complex.

Interestingly, the *P. corrugata pcoR* and *rfiA* mutants and not the *pcoI/rfiA* double mutant showed significant reduced virulence when inoculated in tomatoes. Moreover, CLPs were absent in the cultural filtrates of the *pcoR*, and *rfiA* mutants whereas the double *pcoI/rfiA* still produced them (Licciardello *et al*., 2012).

Genetically adjacent to *rfiA* we described an RND-type efflux system, designated *pcoABC* which is under positive regulation by RfiA and, indirectly, by the PcoI/R system (Licciardello *et al*., 2009). The PcoABC system is highly homologous to the PseABC efflux system of *P. syringae* pv. *syringae* B301D, where it has a role in the secretion of syringomycin and syringopeptins (Kang and Gross 2005). A null mutant in *pcoABC* retained the ability to inhibit the growth of CLP indicator microorganisms *R. pilimanae* and *B. megaterium* although at a reduced level compared to the WT and was as virulent as the WT strain, indicating that most probably other CLP secretion mechanism(s) exist in *P. corrugata* (Licciardello *et al*., 2009).

Like many other biologically active secondary metabolites, CLPs are synthesized by multifunctional non-ribosomal peptide synthetases (NRPS) (Raaijmakers *et al*., 2006). NRPS consist of several modules, each having a specific function in the biosynthesis of CLPs and of the peptide antibiotics.

The association of ABC transporter genes with gene cluster of non-ribosomally synthesized peptides, is not uncommon (Méndez and Salas, 2001), and the conserved positioning of these genes suggest an important role in CLP biosynthesis and transport (de Bruijn *et al*., 2008).

P. corrugata aforementioned genes (*pcoI/pcoR; rfiA, pcoABC*) are located on one out of two contigs of a cosmid insert of approximately 20,000 bp; the sequence of the

whole insert support the presence of genes putatively encoding for an additional ABC transporter and a part of a non-ribosomal peptide synthetase highly homologous to genes in the CLP biosynthetic cluster in other *Pseudomonas* spp. Therein we demonstrate that this ABC transporter form an operon with the last gene of a putative NRPS and that both are part of the biosynthesis cluster of *P. corrugata* corpeptins, which we show have an important role in virulence and antimicrobial activity of *P. corrugata* The role of PcoI/PcoR QS system and the RfiA regulator in the regulation of the expression of these genes was also demonstrated.

4.3. Materials and Methods

4.3.1. Bacterial strains and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 5. *Pseudomonas corrugata* strains and *Chromobacterium violaceum* strain CV026 (AHL bacterial biosensor) were routinely grown at 28°C in Nutrient Agar (Oxoid, Milan, Italy) plus 1% dextrose (NDA) or in Luria-Bertani (LB) agar. *Escherichia coli* strain DH5α (Hanahan, 1983), and CC118λ*pir* were used as hosts for the plasmids and for insertional mutagenesis. *E.coli* strains were grown at 37°C on Luria-Bertani (LB) plates or in LB broth. Antibiotics were added as required at the following final concentrations: ampicillin 100 μg ml^{-1}; kanamycin 50 μg ml^{-1} (*C.violaceum*) or 100 μg ml^{-1}; tetracycline 15μg ml^{-1} (*E. coli*) or 40 μg ml^{-1} (*Pseudomonas* spp.).

4.3.2. DNA recombinant techniques

DNA manipulations, including digestions with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments, ligation with T4 ligase, DNA hybridization, radioactive labeling by random priming, and *E.coli* transformation were performed as described by Sambrook *et al* (1989). Southern hybridizations were performed by using N+Hybond membranes (Amersham Biosciences); plasmids were purified by the alkaline lysis method (Birnboim, 1983); total DNA from *Pseudomonas* spp. was isolated using the Gentra Puregene Cell Kit (Qiagen). Triparental matings from *E. coli* to *P. corrugata* were carried out with the helper strain *E. coli* DH5α (pRK2013) (Figuraski & Helinski, 1979).

Table 5 - Bacterial strains, plasmids and oligonucleotides used in this study

Strains	Genotype/relevant characteristics	Reference or source
P. corrugata		
CFBP 5454	Wild type, source of *corpC* and *corpD*	CFBP[a]
PCOMFP	*crpD*:: pKnock, Km^r	This study
PCONRPS	*crpC*:: pknock, Km^r	This study
GL1	*pcoI*362::Tn5, Km^r	Licciardello *et al* (2007)
GL2	*pcoR*76::Tn5, Km^r	Licciardello *et al*. (2007)
GLRFIA	*rfiA::* pKnock, Km^r	Licciardello *et al*. (2009)
E. coli		
pLC3.34	pLAFR3 containing *P. corrugata* CFBP 5454 DNA, Tc^r	DISPA
DH5α	F2 f80d*laczZDM15* D(*lacZYA-argF*)*U169 endA1 recA1 hsdR17 deoR gyrA96 thi-1 relA1 supE44*	Sambrook *et al*. (1989)
CC118 λ *pir*	Δ (*ara, leu*)*7697 araD139* Δ *lacX74 galE galK phoA20 thi-1 rpsE rpoB* (Rf^r) *argE*(Am) *recA1* λ *pir*	Herrero *et al*. (1990)
C.violaceum		
CV026	ATCC 31532 derivative, *cvil*::Tn5*xylE* Km^r Sm^r	McClean *et al*.(1997)
Plasmids		
pCR2.1	Cloning vector TA, Amp^r	Invitrogen
pKNOCK-Km^r	Mobilizable suicide vector, Km^r	Alexeyev (1999)
pRK2013	Km^{r}100, Tra$^+$ Mob$^+$ ColE1 replicon	Figurski and Helinski, (1979)
pKMMfp	pKNOCK containing an internal fragment of *P. corrugata* CFBP 5454 *crpD* gene	This study
pKMNrps	pKNOCK containing an internal fragment of *P. corrugata* CFBP 5454 *crpC* gene	This study
Oligonucleotides	**Sequence**	
MFPkn-fw	5'- AAGGATCCAGTGGCTGGCGGAAATC - 3'	This study
MFPkn-rev	5'- GGTCTAGAGGATGGTGAAATACACT - 3'	This study
NRPSkn-fw	5' – CAGGATCCGGATCTATCTGCTCGAC – 3'	This study
NRPSkn-rev	5'- AATCTAGAGCCGATAGTGCCGAGGG – 3'	This study
PCR1 fw	5'- ACCGCAACATCAATACAGCG - 3'	This study
PCR1 rev	5'- ACCGACATCAACCTGCTTGAC - 3'	This study
PCR2 fw	5' - CATCGCCTGCGTATCTCGAT - 3'	This study
PCR2 rev	5' - CAACTCATGGTCGTCCATCG - 3'	This study
Pco16S fw	5' – TGTAGCGGTGAAATGCGTAGAT -3'	Conte *et al*.(2006)
Pco16S rev	5' – CCTCAGTGTCAGTATCAGTCCAG -3'	Conte *et al*.(2006)
Abc1 fw	5' – CAAAATCGCTATCGTGCTTGTC-3'	This study
Abc1 rev	5' – CGACCGTAGCGGTCAGGTA-3'	This study
nrps fw	5' – ACGGGCCACCCGAAAG – 3'	This study
nrps rev	5' – GAGGCGAAAGCCACGTGAT -3'	This study
Primer seq fw	5' - GATCCATCGACGGACTGTC – 3'	This study
Primer seq rev	5' - CATCGTGTTCGGTTTCGTAC -3'	This study

[a]CFBP, Collection Francaise de Bacteries Phytopathogenes, Angers, France.

4.3.3. DNA sequencing and sequence analysis

Two contigs were obtained sequencing the cosmid pLC3.34 DNA insert on both strands (Macrogen, Inc, Seoul, Korea). A set of primers overlapping the ends of the two sequence were used to merge the gap between the two contigs yielding a 2284 bp amplicon (Table 5). The resulting amplicon was sequenced on both strands by BMRCRIBI (University of Padua, Italy). The open reading frames (ORFs) were identified with the ORF Finder graphical analysis tool and homology searches of nucleotide and protein sequences were performed using the BLAST searching program BlastX and BlastN in the NCBI database. (Altschul *et al.*, 1990). For phylogenetic analysis, alignment were made with ClustalW incorporated into Mega5.0 software package (Tamura *et al.*, 2011), and trees were inferred by neighbor joining using 500 bootstrap replicates. Trans-membrane segments (TMS) of CrpE and of homologous putative CLP transporter from other *Pseudomonas* spp. were predicted using the Membrane Protein Explorer (MPex) tool by Jayasinghe *et al.* (2001) at http://blanco.biomol.uci.edu/mpex. This sliding-window hydrophobicity analysis of amino acid sequences of membrane proteins is useful to identify putative trans-membrane parts.

The previous sequence Accession EF189721 was thus substituted with the complete insert with the Accession number KF192265.

4.3.4. Cell free culture filtrate and RNA sample preparation

P. corrugata CFBP 5454 and derivative mutants cell free culture filtrates were used to assess AHL and CLP production and to test all strains for biosurfactant activities. Bacterial strains were grown in IMM (Surico *et al.*,1998) at 28°C for 4 days. *P.corrugata* CFBP 5454 was also grown in IMM supplemented with 5µM of synthetic AHL (C_6-HSL and C_8-HSL). Time course analysis of the *pcoI/rfiA* mutant and parent strains was determined by sampling aliquots of cultures incubated in static condition from triplicate flasks 17, 24, 48,72, 96 hour post inoculation. After centrifugation (9000 × *g*, 20 min), the supernatant was passed through a 0.22-µm Millipore filter (Millipore, Billerica, MA, U.S.A.) to obtain cell-free culture filtrates. Aliquots of all samples were lyophilized and resuspended in sterile water to obtain a 10X cultural filtrate.

Total RNA was prepared from *P. corrugata* CFBP 5454 and mutant strain cells, sampled at specific time points from aliquots of the cultures grown as described above,

using the RNeasy Mini kit (Qiagen Inc., Valencia,CA) according to manufacturer's directions.

4.3.5. Construction of *P. corrugata crpD* and *crpC* knock-out mutants

The central part of *crpD* and *crpC* genes were amplified by PCR as a 486-bp and 1234-bp fragments, respectively, using the primers MFPkn-fw and MFPkn-rev, NRPSkn-fw and NRPSkn-rev (Table 5). These fragments were first cloned into pCR2.1 vector (Invitrogen, Milan, Italy) according to the manufacturer's instructions, and then subcloned by *Bam*HI/*Xba*I digestions into pKNOCK-Km suicide vector, generating PKMMFP and PKMNRPS respectively. These latter plasmids were transferred into *P. corrugata* CFBP 5454 by triparental mating, generating PCOMFP and PCONRPS. Transformants were selected on LB agar plates supplemented with kanamycin 100 µg ml^{-1} and confirmed by Southern Blot analysis.

4.3.6. Reverse transcription polymerase chain reaction

RT-PCR analysis was performed to define that *crpC*, *crpD* and *crpE* are transcriptional joined. Two sets of specific primers Nrps fw-Abc1 rev (PCR1) (523bp) and Abc1fw-Abc2 rev (PCR2) (512bp) (Table 5) were used to identify the putative transcripts overlapping respectively the *crpC-crpD* and the *crpD-crpE* regions. Following a DNAse purification step by DNAse I (Invitrogen) one microgram of RNA was used for cDNA synthesis with Superscript III (Invitrogen) according to the manufacturer's protocol. Genomic DNA was used to test the fidelity of the primer pairs, whereas samples in which reverse transcriptase was not added were used as negative controls. PCR reaction were performed using a Gene Amp PCR system 9700 (PE Applied Biosystem, Milan, Italy) under the following conditions: an initial 94°C for 2 min; followed by 35 cycles of 94°C for 45 sec, 56°C for 45 sec and 72°C for 45 sec; and a final extension of 72°C for 5 min. The RT-PCR products were subjected to electrophoresis with a 1.5% agarose gel

4.3.7. Transcriptional analysis by Quantitative real-time PCR (Q-PCR)

To determine whether *crpD* and *crpC* expression is regulated by Quorum Sensing system PcoI/PcoR and the transcriptional regulator RfiA, WT strain *P. corrugata* CFBP 5454 and derivative mutants were analyzed by Q-PCR. The same technique was also used to

determine *crpD*, and *crpC* transcript levels in PCOMFP and PCONRPS strains. The primers for the Q-PCR used are listed in Table 5 (Abc1 fw-rev for *crpD*, and nrps fw – rev for *crpC*). Reactions were conducted with the BioRad iQ5 Cycler and the SYBR GreenER qPCR Super Mix iCycler **(**Invitrogen**)** according to the manufacturer's protocols. To correct small differences in template concentration, the 16S rRNA gene was used as housekeeping gene (Conte *et al.*, 2006). The cycle where the SYBR green fluorescence crosses a manually set threshold cycle (C_T) was used to determine transcript levels. The C_T value for each gene was corrected for the housekeeping gene as follows: $\Delta C_T = C_T$(gene X) – C_T (*16S rRNA*). The relative expression (RE) values were calculated by the formula $RE = 2^{-[\Delta CT(\text{mutant}) - \Delta CT(\text{wild type})]}$ (Livak and Schmittgen; 2001). If there is no difference between mutant and wild type, then RE = 1 (2^0). Q-PCR analysis was performed in duplicate on three independent RNA isolations.

4.3.8. *In vitro* bioassay for AHL and CLP production and surface tension measurement

P. corrugata CFBP 5454 and respective mutants cultural filtrates were used to asses AHL and CLP production. Culture filtrate of *P. corrugata* CFBP 5454 were added to the AHL CV026 biosensor to determine AHL production by visual detection of violacein formation, which is dependent upon the external addition of medium-chain-length lactones (Martinelli *et al.*, 2004; Licciardello *et al.*, 2009). Antimicrobial activity was assessed by well-diffusion assay in plates containing a double layer of solidified potato dextrose agar (PDA) (Oxoid) containing the two indicator strains *Rhodotorula pilimanae* ATTC26432 and *Bacillus megaterium* ITM100. (Licciardello *et al.*, 2009). After the addition of 5% methylene blue (which is useful for photography but has no influence on droplet surface tension), 10 µl of each strain was pipetted as a droplet onto Parafilm and the spreading of the droplet was observed (Vallet-Gely *et al.,* 2010). All tests were carried out twice in triplicate each time.

4.3.9. MALDI-TOF analysis

The samples were dissolved in a solution of 49.9% water, 0.1% Trifluoroacetic Acid and 50% CH_3CN before to be analyzed through the mass spectrometer. MALDI-TOF- mass spectrometry experiments were carried out on a Voyager DEPRO mass spectrometer (PerSeptive Biosystems, Framingham, MA) equipped with an N2 laser (337 nm, 3 ns pulse

width) operating both in the linear and reflector positive ion modes using the Delayed Extraction. Samples were co-crystallised with 3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enoic acid (sinapic acid) as the matrix, which was prepared by dissolving 10 mg of sinapic acid in 1 mL of aqueous 50% (v/v) acetonitrile containing 0.1% (v/v) Trifluoroacetic Acid. Raw data were elaborated using the software program Data Explorer version 4.0 (Applied Biosystems).

4.3.10. Plant inoculations

P. corrugata CFBP 5454 and derivative mutants were tested for pathogenicity on tomato CV Marmande plants grown in nursery flats, one month after germination. During the trials, plants were maintained in a growth chamber with 16/8 h photoperiod and 26°C temperature. Tomato plants were pin-prick on the stem at the axil of the first true leaf (20 plants per strain) with bacterial cells from 48-h culture on NDA. After inoculation, plants were enclosed in polyethylene bags to maintain 100% relative humidity for 3 days; the bags were then removed until the end of the experiment. Plants were rated for symptoms 15 days after inoculation: the stem of each plant was cut longitudinally and the length of pith necrosis measured.

4.4. Results

4.4.1. *crpD* and *crpE* that encode for a type I secretion system and the NRPS synthetase gene *crpC* are part of corpeptin biosynthetic locus

Analysis of potential CLPs biosynthesis gene clusters of various *Pseudomonas* spp. revealed a conserved structure and include NRPS, LuxR-type transcriptional regulators, ABC transport carriers, and an RND-like outer membrane proteins (Raaijmakers *et al.*, 2010).

P. corrugata produces the antimicrobial and phytotoxic CLPs: corpeptins (CPs) A and B (CP-A and CP-B) and cormycin A (CM-A) (Emanuele *et al.*, 1998; Scaloni *et al.*,2004). At present there is no information regarding their biosynthesis, nevertheless recently we demonstrated that the AHL QS system of *P. corrugata* PcoI/PcoR and the transcriptional

regulator RfiA have an important role in regulation of virulence via CLP production (Licciardello *et al.*, 2012). The absence of further genetic and molecular information prompted us to sequence the complete cosmid insert from which the AHL QS genes were identified in order to gain insight of the adjacent genes of these CLP regulators. The cosmid pLC 3.34 is part of the genomic library of *P. corrugata* strain CFBP5454 in pLAFR3. DNA sequencing of the pLC3.34 insert revealed two contigs and the intervening region between the two contigs was amplified to determine the overlapping sequences. Sequencing on both strands of this amplicon allowed reconstructing the entire sequence of approximately 21 Kb of the cosmid pLC3.34 insert DNA (Fig. 19).

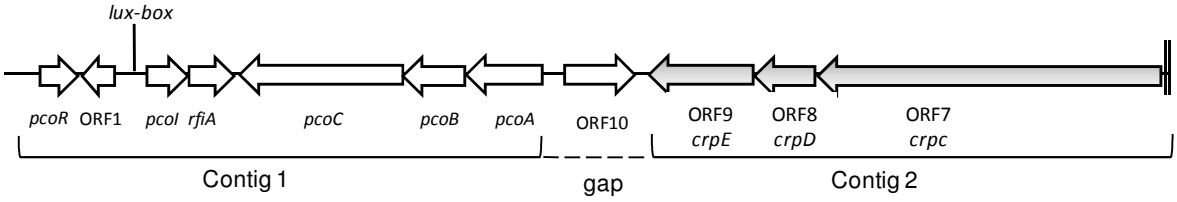


Fig. 19 - Representation of contig assembly of an approximately 21-Kb DNA region of *Pseudomonas corrugata* CFBP 5454. The first contig (10 Kb) harbors the AHL-QS system genes (*pcoI/pcoR*), the transcriptional regulator *rfiA* belonging to the LuxR type transcriptional regulators and *pcoABC* operon, encoding for a resistance nodulation-cell-division transporter system. The second contig (11 Kb) contains the corpeptin genes *crpC, D* and *E*. The gap region represents an approximately 2.3 Kb DNA sequence obtained by PCR using a set of primers overlapping the ends of the two contings.

Three complete and one partial new ORFs were identified: ORF8 spans 1152 bases and encoded for a putative 383-amino acid protein displaying high homology with members of the periplasmic protein component of membrane transporters. It displayed 81% identity (91% similarity) with a putative macrolide efflux protein MacA identified in *P. fluorescens* Pf-5 (YP_259255.1). Downstream of this ORF, separated only by 2 bp, and transcribed in the same direction ORF9 spans 1962 bases and encoded a predicted 653-amino acid protein which was 81% identical (90% similar) to an ABC transporter of *P. syringae pv. syringae* B728a (YP235695). Translated in the opposite direction 299 bp downstream of the ORF9, we identified a putative diaminobutyrate -2-oxoglutarate transaminase protein (ORF10). It displayed 80% identity to *P. syringae* B728a

(YP_235696.1). Located 78-bp upstream of the ORF 8 and translated in the same direction, we identified a truncated ORF (ORF 7). These 6471 bases encode 2156-amino acids that showed high homology with members of non-ribosomal peptide synthetases (NRPS) involved in CLP biosynthesis gene clusters of other *Pseudomonas* spp. For example, this partial ORF has 62% identity (76% similarity) with the arthrofactin synthetase C identified in *Pseudomonas spp*. MIS38 (BAC67536.1); 61% identity (74% similarity) with massetolide MassC protein of *P. fluorescens* (ABH06369.2) and 63% identity (76% similarity) with orfamide non-ribosomal peptide synthetase OfaC of *P. fluorescens* Pf-5 (YP_259254.1).

Conserved domain analysis of ORF 7 protein revealed the presence of an entire amino acid activation module, containing conserved core sequences for the condensation, amino acid activation, and thiolation domains. Part of another amino acid activation module and two thioesterase TE domains each containing the conserved GxSxG sequence motif involved in the linear or cyclic peptide release were also identified.

In order to examine the role of these newly identified ORFs in CLP biosynthesis, mutants in which the genes encoding ORF7 and ORF 8were insertionally inactivated creating two genomic mutants of *P. corrugata* designated PCONRPS and PCOMFP respectively (Table 5). *P. corrugata* strain CFBP 5454 produces two types of CLPs; MALDI-TOF MS analysis of the cell free cultural filtrates revealed the presence of CM-A and CPs corresponding to specific peaks at *m/z* of 1274 and *m/z* 2095.3 and 2121.2 (Licciardello *et al*., 2012). The analysis of the cell free culture filtrates of the mutants in ORF7 and ORF8 grown in CLP inducing conditions as compared to the WT strain revealed that the two mutants no longer produced CPs, whereas still produce CM-A (Fig. 20). On the basis of genotypic and phenotypic evidence we designated ORF7, ORF8 and ORF9 as *crpC*, *crpD* and *crpE* respectively and PCOMFP and PCONRPS the *crpD* and *crpC* mutants, respectively. The results led us to postulate that CrpD and CrpE were probably involved in the secretion of CPs and that CrpC is part of the NRPS cluster for the biosynthesis of this CLP (Fig. 20).

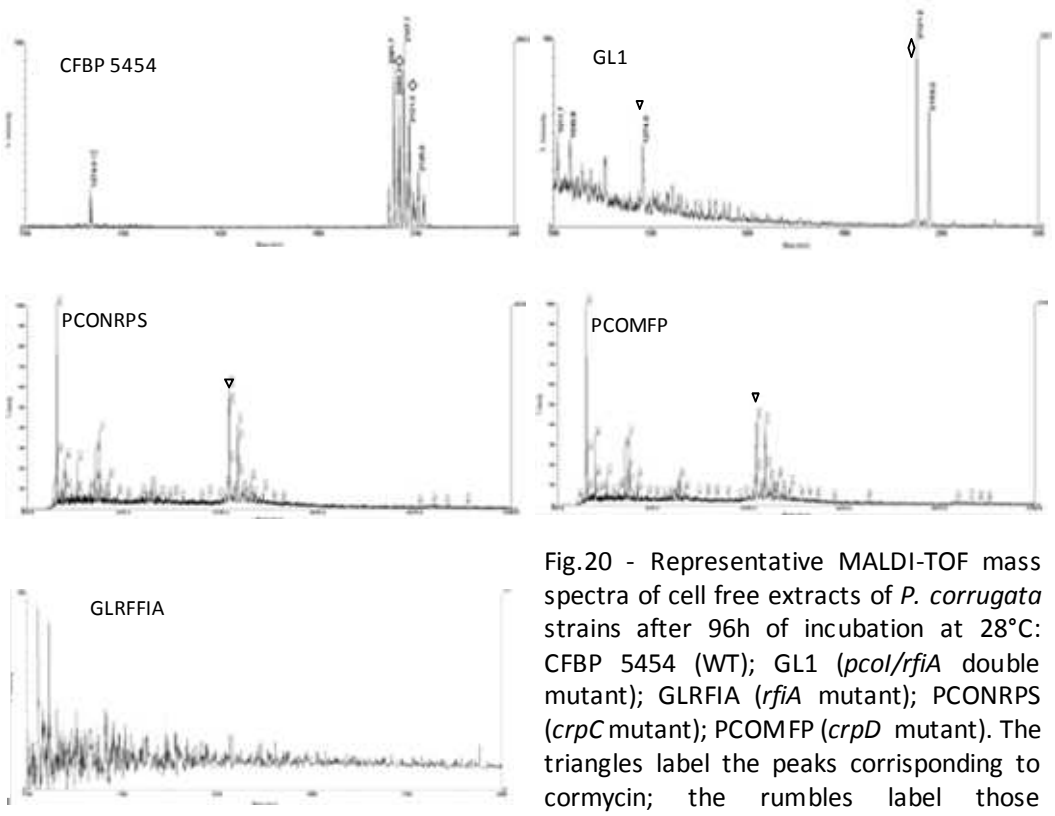


Fig.20 - Representative MALDI-TOF mass spectra of cell free extracts of *P. corrugata* strains after 96h of incubation at 28°C: CFBP 5454 (WT); GL1 (*pcoI/rfiA* double mutant); GLRFIA (*rfiA* mutant); PCONRPS (*crpC* mutant); PCOMFP (*crpD* mutant). The triangles label the peaks corrisponding to cormycin; the rumbles label those corresponding to corpeptins.

4.4.2. The *crpCDE* are part of an operon

In order to determine if *crpCDE* constituted an operon, we used a reverse transcription approach with two sets of primers overlapping the *crpD* and *crpE* genes and the *crpC* and *crpD* region (Fig. 21A). PCR1 primer set was designed in order to capture an intervening region between *crpD* and *crpE* , upstream the knock out inactivation site of *crpD*. The WT and PCOMFP (*crpD*⁻*)* strains produced the PCR1 specific amplicon, whereas it was absent in the PCONRPS strain (*crpC*⁻*)*, indicating that the two ORFs were co-transcribed. To demonstrate that *crpC* and *crpD* were in an operon, PCR2 primer set were used. As expected, the WT strain yielded the predicted 512 bp PCR2 product, no amplification product was observed in the two mutant strains (Fig. 21B). It was concluded that the genes downstream the mutation in the PCONRPS strain (*crpC*⁻) were also inactive, thus *crpCDE* constituted an operon.

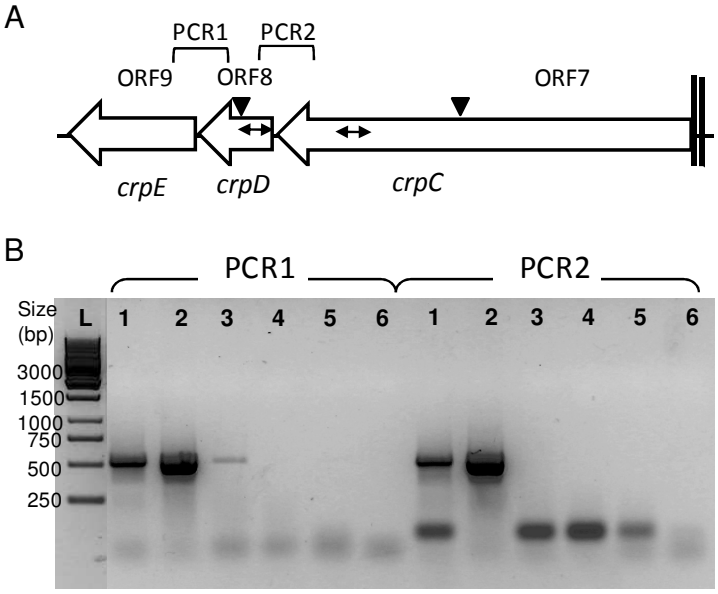


Fig.21 - (A) Polycistronic organization of *crpCDE* cluster identified in *P. corrugata* CFBP 5454. Triangles represent the position of insertional mutagenesis. The brackets indicate the position of primer sets overlapping respectively *crpD* and *crpE* (PCR1) and *crpC* and *crpD* (PCR2). For *crpC* and *crpD* double arrows indicate the location of the primers used for quantitative real-time PCR (Q-PCR). (B) Agarose gel electrophoresis of RT-PCR analysis demonstrating the polycistronic transcription of *crpC*, *crpD* and *crpE*. RT-PCR analysis was performed with total RNA isolated from *P. corrugata* CFBP 5454 and mutant strains incubated on IMM for 4 days at 28°C. Two sets of primers (PCR1 and PCR2) were designed for amplifying approximately 500 bp DNA fragments at each inter-gene locus, gap between *crpC* and *crpD* and between *crpD* and *crpE*. L: ladder; 1 : WT; 2: genomic DNA WT; 3: c*rpD* mutant; 4: *crpC* mutant; 5: negative control of the reverse transcription step; 6: negative control water.

Quantitative real-time PCR (Q-PCR) assays showed that *crpC*⁻ transcription occurred in the PCOMFP strain (*crpD*⁻), whereas it was strongly reduced in the PCONRPS strain (*crpC*⁻) . *crpD* expression was detected in the WT strain and PCOMFP strain (*crpD*⁻), since the primers were designed upstream the mutation but was undetectable in the PCONRPS strain (*crpC*⁻), confirming that the three ORFs were transcriptionally joined as already observed by RT-PCR (Fig. 22A). The results collectively support the role for CrpD/E as corpeptin CLP transporter, furthermore the expression of the putative corpeptin synthetase gene in the PCOMFP (*crpD*⁻) strain suggest that corpeptins were produced by the PCOMFP (*crpD*⁻) strain but most probably not released outside the cell.

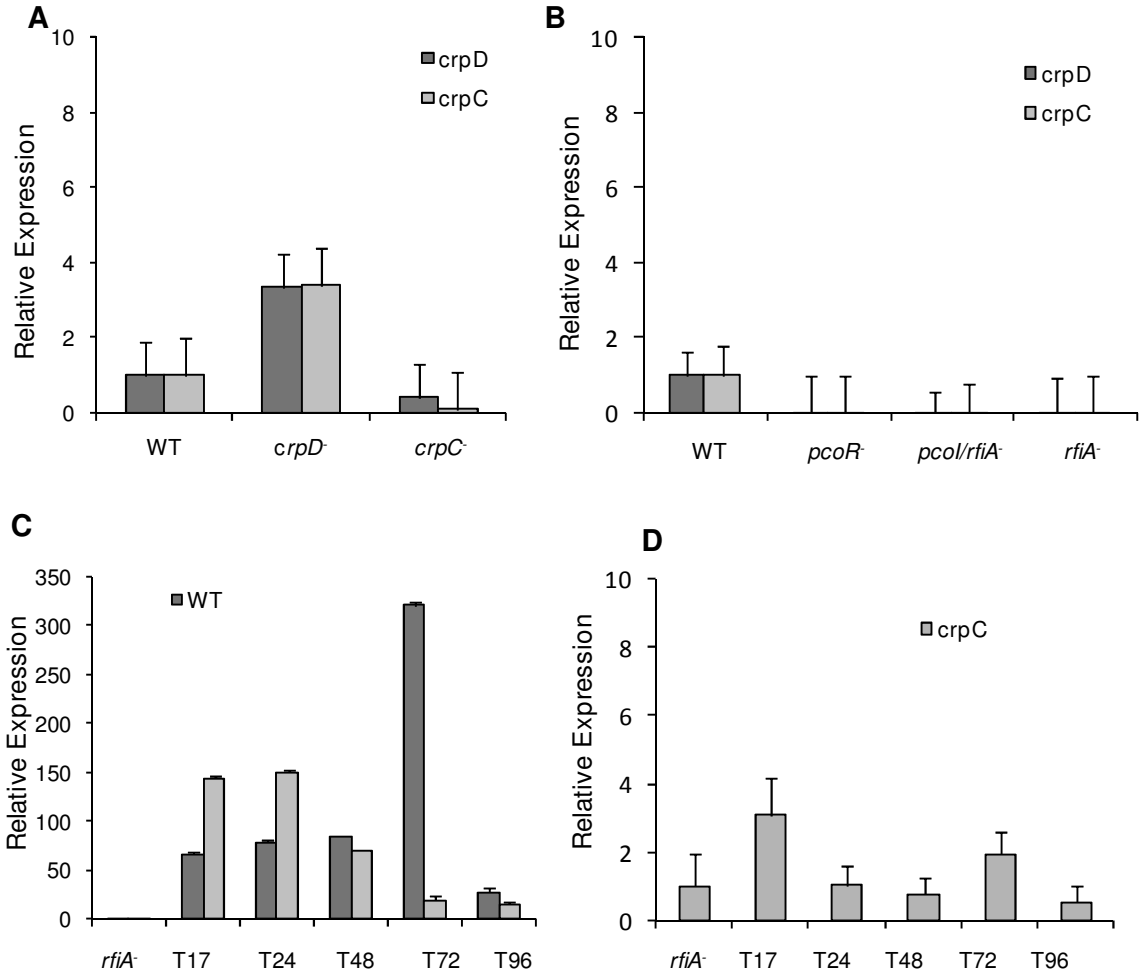


Fig. 22 – Transcriptional expression analysis. Gene expression was determined on cDNAs of cultures incubated in IMM at 28°C either for 96 h (A and B) or time course (in C and D). Expression levels were normalized using the *16S* rRNA gene. Expression of *crpD* and *crpC* in *crpD* and *crpC* mutants (A) and in Quorum Sensing mutants (*pcoR*$^-$*;pcoI/rfiA*$^-$*, rfiA*$^-$) (B); results are reported as the fold difference relative to data obtained for the parent strain. Time-course expression of *crpC* in *P. corrugata* CFBP 5454 grown either in IMM or IMM supplemented of synthetic AHLs (C) and in the double mutant *pcoI/rfiA*$^-$ grown in IMM (D) results are reported as the fold difference relative to data obtained for non-producing corpeptins mutant *rfiA*$^-$. Mean values are calculated from duplicate sample from two separate experiments. Error bars represent ± one standard deviation from the mean.

4.4.3. Corpeptins are involved in *P. corrugata* biological activity

To investigate the role of CLPs in *P. corrugata* biology we compared a number of phenotypic traits of the PCOMFP (*crpD*$^-$) and PCONRPS (*crpC*) mutant strains (producing

only CM) to the parent strain CFBP 5454 that produce both CM and CPs and to GLRFIA (*rfiA*$^-$) strain (no CLP producing strain). Tomato plantlet stems were inoculated to determine the importance of corpeptins in bacterial virulence. Stem– pith– infected tissue of plants inoculated with the parent strain fifteen days after inoculation appeared dark brown, necrotic and hollowed with ladder-like lesions ranging in length from 3 to 6 cm (Fig. 23A). Most of the plants inoculated with PCOMFP (*crpD*$^-$) and PCONRPS (*crpC*) strains, showed brown discoloration inside the stem considerably reduced in length (approximately 1-2 cm) compared to that caused by the parent strain. The *rfiA*$^-$ mutant did not cause lesions on tomato stem pith other than a discoloration at the inoculation site. This is in accordance with previous results where it was postulated that in absence of CLP toxins the symptoms are greatly reduced if not abolished in this mutant (Licciardello *et al*., 2009; 2012). Antimicrobial activity of culture filtrates of strain producing both CLPs (WT) and of strains able to produce only CM (PCOMFP (*crpD*$^-$) and PCONRPS (*crpC*$^-$)) was tested by a bioassay based on inhibition of *in vitro* growth of *Rhodotorula pilimanae* and *Bacillus megaterium*. PCOMFP (*crpD*$^-$) and PCONRPS (*crpC*$^-$) mutant culture filtrate inhibitory activity was significantly reduced by 100% and 60% against *R. pilimanae* and 60% and 80% against *B. megaterium* respectively compared to the parental strain (Fig. 23A). These findings clearly demonstrated the importance of the ABC transporter system CrpD/E and of the CrpC peptide synthetase, and therefore of corpeptins, in the development of disease symptoms and antimicrobial activity.

4.4.4. PcoI/PcoR AHL-QS system and the transcriptional regulator RfiA regulate *crpCDE* expression

The role of QS in the regulation of *crpCD* expression was investigated by Q-PCR. We previously reported that *P. corrugata* mutations in the transcriptional regulators *pcoR* (GL2 mutant) and *rfiA* (GLRFIA mutant) no longer produced CLPs. Both toxins were however produced by the double mutant *pcoI/rfiA*$^-$ (GL1) although at a reduced level compared to the parent strain(Licciardello *et al*., 2012). We observed that *crpC* and *crpD* transcript levels were significantly and consistently decreased both in the *pcoR*$^-$ and *rfiA*$^-$ mutants as well as in the *pcoI/rfiA*$^-$ double mutant (Fig. 22B). Taken together, these results suggest that, under the conditions tested, AHL QS and RfiA play a role in corpeptin biosynthesis and secretion in *P. corrugata*. Since in the cell free culture filtrate of the

pcol/rfiA$^-$ double mutant both CLPs were detected by MALDI TOF analysis (Fig. 20) we hypothesized that if CLP genes were expressed much earlier than the sampling time (96 h) the mRNA could be degraded, compromising gene expression detection. We therefore performed a time course batch in order to monitor the time of growth and the toxins production/secretion.

Time course cultures (T17-T24-T48-T72-T96) of the WT *P.corrugata* and *pcol/rfiA*$^-$ mutant strains were performed and investigated for the presence of transcript levels of *crpC*; corresponding culture filtrates were also tested for AHL production, antimicrobial, biosurfactant activity and also performed MALDI-TOF analysis. During the growth of the WT strain, transcript levels of *crpC* did not change until T48, reaching a maximum at T72 (320 folds) and decreased consistently at T96 (Fig. 22C). AHL presence, detected indirectly by violacein production of the biosensor *Chromobacterium violaceum* CV026 cultures, was first detected in the culture filtrates at T48 with cell concentration as low as $7{\cdot}10^8$ CFU ml^{-1} (Fig. 23C). Transcription of *crpC* was anticipated by the addition of exogenous AHLs at a concentration of 5 μM to early-log-phase cultures (OD_{600} 0.2) of *P. corrugata* (Fig. 22C). Culture filtrates, showed antimicrobial activity against *R. pilimanae* and *B. megaterium* (Fig. 23B) and were able to decrease the surface tension of the culture filtrate as assessed by "drop collapse assay" starting from T72 (Fig 5C).

The *crpC* transcription in the double mutant *pcol/rfiA*$^-$, on the other hand was of limited extent (3 folds) and confined to the initial step of growth (Fig. 22D). Moreover MALDI-TOF MS spectra showed that the *pcol/rfiA*$^-$ mutant strain produced very low amounts of CLPs compared to the parent strain (Fig. 20). No antimicrobial activity *in vitro* was detected at all time points (Fig. 23B).

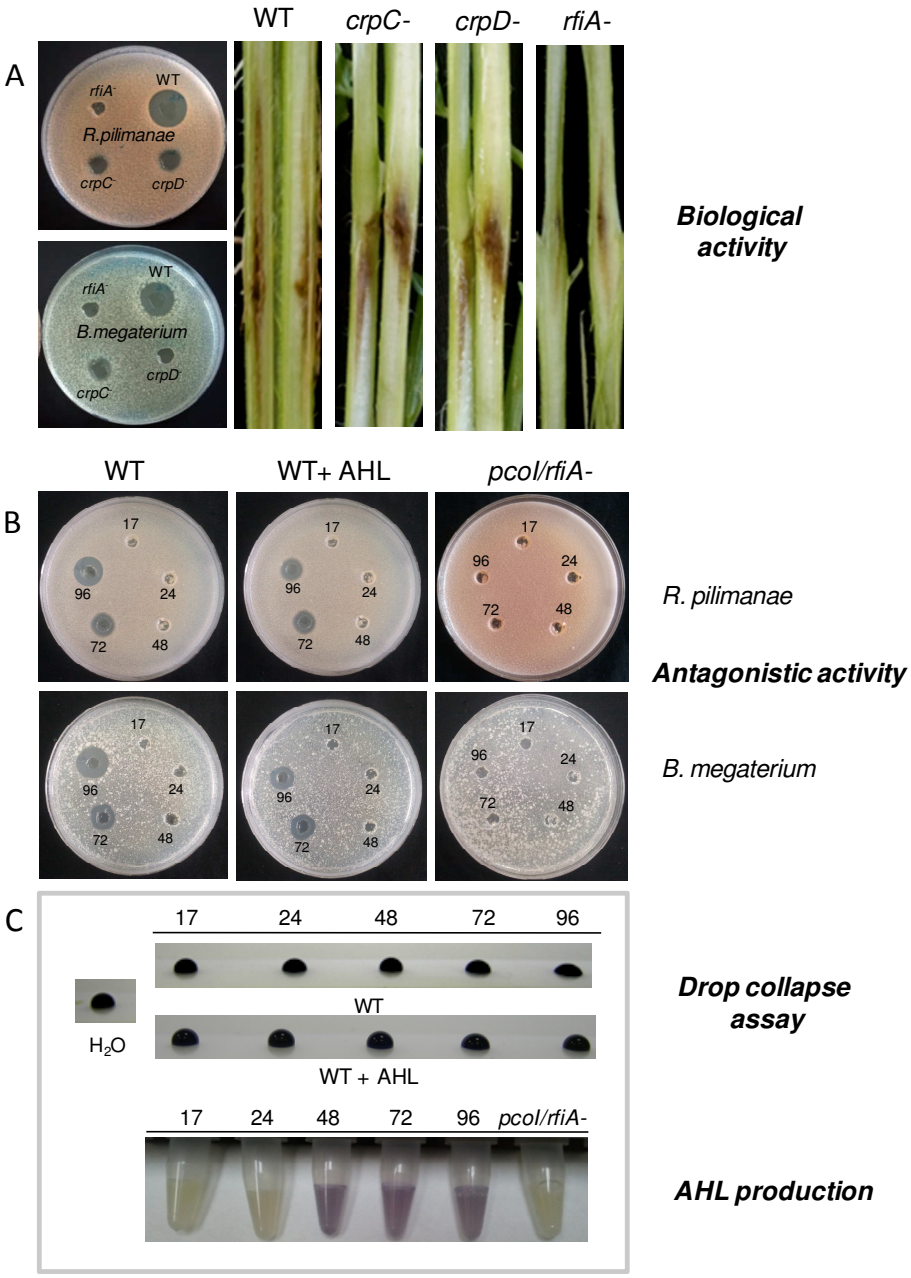


Fig. 23 - Biological activity of the parent strain *P. corrugata* CFBP 5454 and PCOMFP (*crpD* mutant) and PCONRPS (*crpC* mutant). (A): antagonistic activity of culture filtrates (10X) against the CLP indicator microorganisms *Rhodotorula pilimanae* and *Bacillus megaterium*; stem pith necrosis symptoms in prick inoculated tomato plants; *rfiA*$^-$ mutant strain was used as non-producing CLP strain control. (B) Time course biological activity of *P. corrugata* CFBP 5454 and GL1 (*pcoI/rfiA* double mutant) free cell culture filtrates: antagonistic activity, drop collapse activity and AHL production at 17-96 hour post inoculation (hpi) in IMM at 28°C.

4.4.5. CrpE is phylogenetically related to other putative "peptins" trasporters

BLASTx analysis of CrpE revealed homologies with a number of ABC transporters of *Pseudomonas sensu stricto* species with a similarity ranging from 90% to 85%. *P. corrugata* CrpE sequence analysis showed that the N-terminal half contained a Nucleotide Binding Domain (NBD) fold which is characterized by Walker A (GASGSGKST), Walker B (IILAD) and the linker peptide (C-loop), LSGGQQQRVS. This linker peptide is the signature of ABC transporter family proteins (Schneider and Hunke, 1998). Located at the C-terminal half of this protein contains four putative trans-membrane segments (TMS) (Fig. 24), which probably form a trans-membrane domain (TMD). This structural feature suggested that CrpE belongs to ABC transporter protein group that possess NBD and TMD in a single polypeptide (Biemans-Oldehinkel *et al.*, 2006).

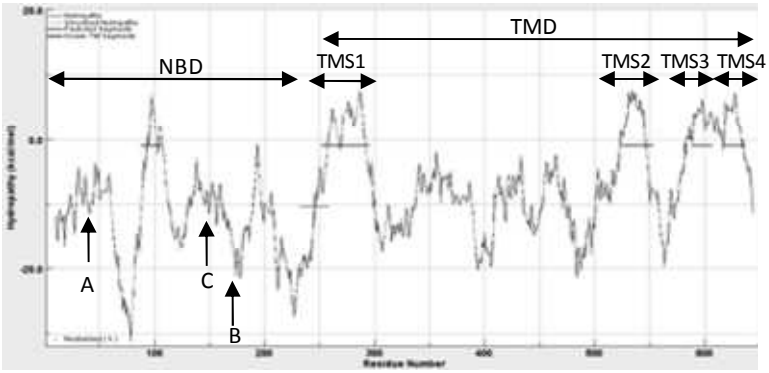


Fig. 24 - Hydropathy plot of CrpE. Abbreviations are NBD, nucleotide binding domain; TMD, transmembrane domain; TMS, transmembrane segment; A, Walker A; B, Walker B; C, C-loop.

Sequence analysis of those of the plant associated *Pseudomonas* evidenced they contained the conserved sequence motifs (Walker A, WalkerB and C-loop) and from 4 to 6 trans-membrane segments (Table 6). The genomic context of each of the ABC "hits" was examined and their encoding genes were all localized downstream of putative CLP synthetase genes (Table 6).

Table 6 - Structural characteristics of CrpE homologous ABC transporters in plant associated *Pseudomonas*

Strain	Protein Accession	Description	Biosynthesis gene cluster	Identities	Similarity	Walker A Walker B C-loop	Number of TMS	Reference
P.fuscovaginae UPB0736	ZP_10991311.1	ABC transporter	Fuscopeptin?? (not described)	81%	90%	Yes	4	Patel et al., unpublished
P. syringae pv. *syringae* 642	ZP_07265130.1	ABC transporter	Unknown	81%	90%	Yes	5	Clark et al., 2010
P. syringae pv. *syringae* B728a	YP_235695.1	ABC transporter	Syringopeptin	81%	90%	Yes	5	Feil,H. et al., 2005
Pseudomonas syringae pv. *avellanae* ISPaVe013	ZP_17805054	ABC transporter:Protein of unknown function DUF214	Unkwnown	80%	90%	Yes	5	O'Brien et al., 2012
Pseudomonas sp. CMR12a	AFH75324.1	macrolide export ATP-binding/permease protein MacB	Sessilin	79%	89%	Yes	5	D'aes,J., 2012
P. protegens Pf-5	YP_259256.1	ABC transporter permease	Orfamide	78%	89%	Yes	6	Paulsen,I.T. et al., 2005
P. fluorescens Pf0-1	YP_347947.1	hypothetical protein	Unknown	77%	89%	Yes	5	Silby,M.W et al., 2009
P. fluorescens SS101	ABW87988.1	macrolide ABC efllux protein macB	Massetolide	77%	89%	Yes	5	De Bruijin et al., 2008
Pseudomonas sp. MIS38	BAF40423.1	putative ABC transporter protein	Arthrofactin	76%	88%	Yes	5	Lim et al., 2009
P.synxantha BG33R	ZP_10142999.1	Efflux ABC transporter permease ATP_binding protein	Unknown	77%	88%	Yes	5	Loper et al., 2012
P. fluorescens SBW25	YP_002872144.1	macrolide-specific ABC-type efflux carrier	Viscosin	77%	88%	Yes	5	De Bruijin et al., 2007
Pseudomonas sp. CMR12a	AFH75332.1	macrolide export ATP-binding/permease protein MacB	Motilin	76%	89%	Yes	6	D'aes,J.,2012
P. syringae pv. *tomato* str. DC3000	NP_792636.1	syringafactin efflux protein SyfD	Unknown (putative linear peptide)	73%	86%	Yes	5	Buell,C.R. et al., 2003
P. syringae pv. *actinidiae* M302091	EGH63888.1	syringolide efflux protein SyfD	Unknown (putative linear peptide)	72%	86%	Yes	5	Baltrus et al.,2011
P. syringae pv. *lachrymans* M302278	EGH94284.1	syringolide efflux protein SyfD	Unknown (putative linear peptide)	73%	86%	Yes	4	Baltrus et al.,2011
P.syringae pv. *morsprunorum* M302280	EGH09011.1	syringolide efflux protein SyfD	Unknown (putative linear peptide)	72%	86%	Yes	4	Baltrus et al.,2011

The NJ dendrogram of these ABC transporters aligned by Clustal W in the MEGA 5.01 software resulted in distinct clusters (Fig. 25). *P. corrugata* CrpE formed a cluster together with ABC transporters of *P. syringae* pv. *syringae* strains 642, and B728a, located downstream genes codifying for syringopeptin (Scholz-Schroeder *et al.*, 2003) and those of *P. fuscovaginae* UPB 0736, *P. syringae* pv. *avellanae* ISPaVe013, and *Pseudomonas* sp. CMR12a ABCt. A clear cut was also observed for transporters located downstream of NRPS putatively involved in the production syringofactins, that are linear peptides. The remaining ABC transporters belong to the biosynthesis gene clusters of the CLPs arthrofactin (Lim *et al.*, 2009), massetolide (De Bruijn *et al.*, 2008), sessilin (D'aes *et al.*,2011), viscosin (De Bruijn *et al.*, 2007) and orfamide (Gross *et al.*, 2007).

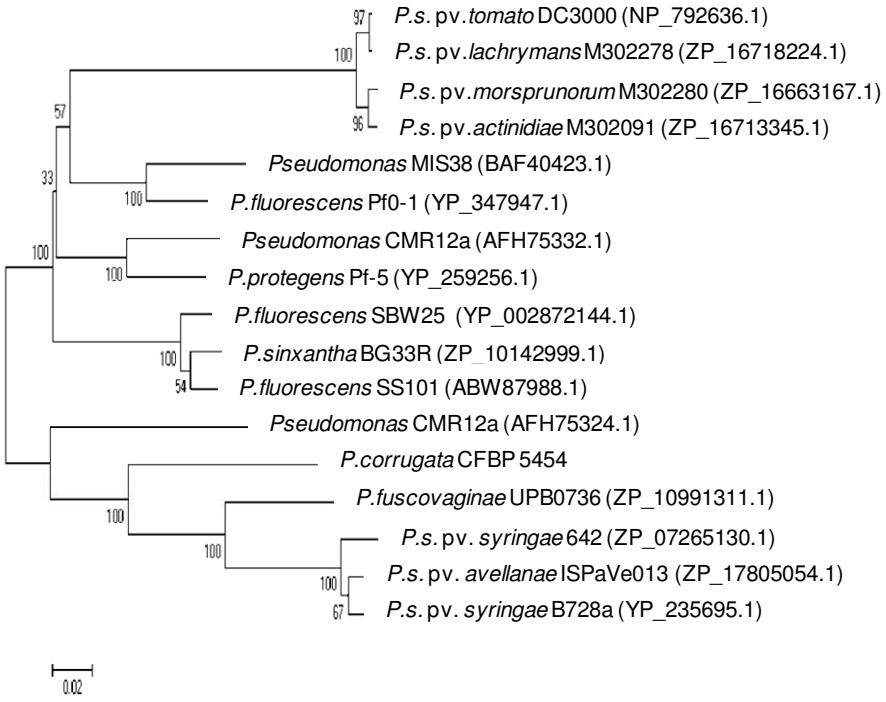


Fig. 25 - Phylogenetic analysis of amino acid sequences of 17 ABC transporter identified in CLP biosynthesis clusters of plant associated *Pseudomonas* spp. The numbers at the nodes indicate the level of bootstrap support, based on neighbor-joining analysis of 500 resampled data sets. The bar indicates the relative number of substitutions per site. In brackets the protein accession number.

4.5. Discussion

CLPs are considered phytotoxins and constitute important virulence factors playing a key role as antimicrobial agents in plant pathogenic and biocontrol *Pseudomonas* spp. Their mode of action occurs via pore formation, followed by cell lysis. They also have an important role in fitness being associated with bacterial motility and biofilm formation (Raaijmankers *et al.*, 2006).

P. corrugata produces the antimicrobial and phytotoxic CLPs, cormycin A (CM-A) and corpeptins A and B (CP-A and CP-B) (Emanuele *et al.*, 1998; Scaloni *et al.*, 2004). We have previously showed that mutants in the transcriptional regulators *pcoR* and *rfiA* were depleted of these metabolites and that CLPs are virulence factors as their production results in increased disease severity (Licciardello *et al.*, 2007; 2009). PcoR is the LuxR homolog of *P. corrugata* QS system PcoI/PcoR whereas RfiA is a LuxR that lacks the AHL binding domain. *rfiA* is intimately linked to QS being co-transcribed with *pcoI* (Licciardello *et al.*, 2009;2012).

In this study we investigated genes found adjacent to these regulators encompassing approximately 10 kb of DNA which we designated *crpC*, *crpD*, *crpE*. Analysis of the 6.4-kb region encompassing the 3′ end of *crpC* indicates that this gene codes for a peptide synthetase dedicated to the biosynthesis of corpeptins via thiotemplate mechanism (Marahiel *et al.*, 1997). The predicted C-terminal portion of the CrpC protein is homologous to proteins that function as NRPS for other CLP biosynthesis. *Pseudomonas* CLPs consist of peptide chains of 7–25 amino acids, thus NRPSs are composed of 7-25 modules codified by several genes, although in some case biosynthesis genes are not physically linked (Gross and Loper, 2009). The mutagenesis of a module is sufficient to disrupt the production of the respective metabolite (Scholz-Schroeder *et al.*, 2001). The introduction of a mutation in *crpC* disrupted a toxin biosynthesis gene and yielded a *P. corrugata* strain that failed to produce corpeptins. Downstream of CrpC, *crpDE* encoded for an ABC transporter system consisting of a cytoplasmic membrane fusion protein (MFP) *crpD* and ABC transporter (ABCt) *crpE* that showed similarity to macrolide efflux systems of other *Pseudomonas* spp. (MacA/B homologs). ABCt in Gram-negative bacteria usually require two accessory envelope proteins for its full function: a specific MFP and an outer membrane protein (OMP) (Wandersman and Delepelaire 2004). All these components are required for the assembling of canonical type I secretion system. The CrpD/E system of *P. corrugata* spp. lacks the OMP component like other putative MacA/B homologs from several *Pseudomonas* strains (Lim *et al.*, 2009; Dubern *et al.*,2008; Vallet-Gely *et al.*, 2010). Gene disruption of *crpD* led to the depletion of corpeptins in the cultural filtrates of *P. corrugata* CFBP 5454. The role of CrpD/E was also defined by QRT-PCR since *crpC* was still expressed in the *crpD* mutant although the

metabolite was not detected in the cultural medium suggesting that corpeptins are produced but not released outside the cell. The lack of corpeptins in the resulting mutant strain also support the assumption that it is the unique transport system involved in corpeptin export. The introduction of mutations in *crpC* and *crpD* also had a relevant effect on the biological activity of *P. corrugata*.

P. corrugata CFBP5454 strain cultural filtrate inhibition of *R. pilimanae* and *B. megaterium* was a result of the production of cormycin and corpeptins. Strain GLRFIA (*rfiA* mutant), and GL2 (*pcoR* mutant), were impaired in CLP production and their cultural filtrates were inactive against both microorganisms (Licciardello *et al.*, 2009). The cultural filtrate of strain PCONRPS (*crpC* mutant), showed reduced activity against both *R. pilimanae* and *B. megaterium;* interestingly cormycin was produced by this strain indicating that this activity was most probably due to this molecule. Cultural filtrate of strain PCOMFP (*crpD* mutant), although still producing cormycin showed a marked reduced effect against *R. pilimanae* and was not able to antagonize *B. megaterium.* Analogously, *P. syringae* pv. *syringae* strain B01D *sypA* mutant that still produce syringomycin (but not syringopeptins) was still able to inhibit the growth of both *B. megaterium* and *G. candidum* (fungus used for the same CLP detection purpose); mutants unable to produce syringomycin did not inhibit the growth of *G. candidum* (Scholz-Schroeder *et al.*, 2001). Three transporter system were associated with the secretion of syringomycin and syringopeptins all located in the *syr-syp* cluster: SyrD, a protein homologous to membrane proteins of the ABC transporter family (Quigley *et al.*,1993), and PseABC, a tripartite transporter system homologous to RND efflux system (Kang and Gross, 2005) and an ABC transporter PseEF (Cho and Kang, 2012). Inactivation of these genes led to the reduction of both CLP toxins production as assessed by *in vitro* test using strain B301D derivative mutant strains. CrpDE is highly similar to PseEF nevertheless it seems the major transporter of corpeptins as shown by MALDI TOF spectra of the *crpD* mutant although a RND efflux system, PcoABC homologous to PseABC could be also involved (Licciardello *et al.*, 2009).

Both mutant strains produced only cormycin but their cultural filtrates had a different activity, nevertheless the two strains have different genotypes and strain PCONRPS (*crpC*-) is unable to produce corpeptins, whereas in strain PCOMFP (*crpD*-) *crpC* is still expressed as observed by Q-PCR supporting that the toxin is produced and most probably not exported out of the cell.

Subsequent determination of the virulence of the respective strain into tomato plantlets established that corpeptins contribute to the virulence of *P. corrugata* since the lesion extent induced into. Similarly in *P. syringae* pv. *syringae* strain B301D derivative mutant strains in *sypA,* in the biosynthesis gene cluster of syringopeptins, and/or in *syrB*,

were reduced in virulence as compared to the parental strain in immature sweet cherry pathogenicity test (Scholz-Schroeder *et al.*, 2001).

Little information is available on other ABC transporters involved in CLP exportation. ABC functional role was demonstrated for *Pseudomonas* sp.MIS 38 (arthrofactin), *P. putida* PCL 1445 (putisolvin), *P.entomophila* (entolysin) and *P. syringae* pv. *syringae* B301D (Lim *et al.*, 2009; Dubern *et al.*, 2008; Vallet-Gely *et al.*, 2010; Cho and Kang, 2012). *P. corrugata crpCDE* genes work as an operon as in *Pseudomonas* strain MIS38 arthrofactin biosynthesis genes encoding *arfABC/DE* (Lim *et al.*, 2009). Most of the genes in the *syr-syp* genomic island of *P. syringae* pv. *syringae* strain B301D involved in CLP syringomycin and syringopeptin biosynthesis are also organized into polycistronic operons (Wang *et al.*, 2006).

Plant associated *Pseudomonas* ABC transporters that showed homologies with *P. corrugata* CrpE are all encoded by genes located downstream the last gene of a NRPS biosynthetic cluster. Phylogenetic analysis clustered these ABC proteins in different groups. Interestingly CrpE form a sub-group with ABC transporters of long chain CLP producing *Pseudomonas syringae* strains. Both the phytopathogen *P. fuscovaginae* and the biocontrol agent *Pseudomonas* sp. CMR 12 also have in their CLP arsenal long chain peptides as the 22-AA CLP fuscopeptin A, and fuscopeptin B (Ballio *et al.*, 1996; Coraiola *et al.*, 2008) and the 18-AA sessilin (D'aes *et al.*, 2011; D'aes, 2012), respectively.

Recent studies have highlighted that several global regulatory mechanisms (GacS/GacA, AHL-QS) and specific transcriptional regulators located in proximity of the CLP biosynthesis genes are involved in the regulation of production in *Pseudomonas* spp. The GacS/GacA two-component regulatory system is involved in the regulation of syringomycin (Bender *et al.*, 1999; Willis and Kinscherf 2004), amphisin (Koch *et al.*, 2002), putisolvin I and II (Dubern *et al.*, 2005), massetolide A, and viscosin (de Bruijn *et al.*, 2007, 2008). It seems to work as a master switch, as *gacA* and *gacS* mutants resulted impaired in CLP production. Only in few *Pseudomonas* strains the role of QS in CLP production was demonstrated, namely in the plant pathogen *P. fluorescens* strain 5064 and saprophytic strain *P. putida* PCL1445, where AHL QS was shown to be involved in viscosin and putisolvin biosynthesis, respectively (Cui *et al.*, 2005; Dubern *et al.*, 2006). It must be noted however that most strain of *P. putida* and *P. fluorescens* do not possesses an AHL QS system.

A pivotal role was demonstrated for the LuxR-type transcriptional regulators positioned up- and downstream of the CLP biosynthesis genes in the biosynthesis syringomycin and syringopeptins (Wang *et al.*, 2006), viscosin and massetolide (de Bruijn and Raaijmakers, 2009a). These regulators contain a DNA-binding domain characteristic of the LuxR family but lacks the autoinducer-binding terminus characteristic of the QS LuxR-family proteins (de Bruijn and Raaijmakers, 2009a).

The PcoI/R QS and the LuxR homolog RfiA in *P.corrugata* play a role in bacterial virulence in tomato, the implication of CLP production was foreseen since derivative mutants in these regulators no longer produced cormycin and corpeptins (Licciardello *et al.*, 2009; 2012). In this study we have demonstrated that synthetase (*crpC*) and transporter (*crpDE*) gene expression is abolished in both transcriptional regulator mutants, confirming their pivotal role in the corpeptin production. Moreover the time course analysis in the parent strain highlighted that corpeptin transcription occurs at the achievement of the *quorum* thus the gene expression follow the AHL signal molecules production trend, reaching high amounts of transcripts as consequence of the positive-feedback regulatory loop.

Different is the case of the AHL synthase mutant of *P.corrugata* GL1 (*pcoI*), which is actually a double *pcoI/rfiA* mutant since the two genes constitute an operon and the mutation in *pcoI* had a polar effect on the downstream regulatory gene *rfiA* (Licciardello *et al.*, 2009). This strain retained virulence similar to that of the parent strain and also produced little amounts of both toxins CM and CPs. Time course analysis indicated that *crpC* expression is restricted to the early stages of growth, probably as consequence of the absence of the positive feed-back loop.

The mutagenesis of *P. corrugata* CFBP 5454 genes in this study and the bioinformatic analysis revealed that *P. corrugata* corpeptins biosynthetic locus is highly similar to those of other CLP-producing *Pseudomonas* and that biosynthesis is governed by NRPS and secretion by an ABC transporter system consisting of an ABC transporter and an accessory membrane fusion protein. Functional analysis revealed that this long chain CLPs as for *P. syringae* syringopeptins are important for virulence and that the export outside the cell requires dedicated transporter systems phylogenetically similar for the long chain length CLPs. *P. corrugata* and *P. syringae* both produce two class of similar CLPs and since *syr-syp* cluster in *P. syringae* represents approximately the 2% of its genome it is of interest and importance to study the biosynthesis and regulation. In fact, the genetics of the production of CLPs also in *P. corrugata* that taxonomically is more related to the fluorescent oxidase positive *Pseudomonas* and to the *P. fluorescens* biocontrol strains than to the *P. syringae* pv. *syringae* plant pathogen. An intricate and interesting CLP regulation network is suggested that at a certain point diverge from that of *P. syringae* where the CLP production is regulated via-*SalA* by the GacS/GacA two component system regulator and in *P. corrugata* by the SalA homologous, RfiA, via QS.

5. Role of secondary metabolites in *Pseudomonas corrugata* and *P. mediterranea* biocontrol activity

5.1. Abstract

Bacterial strains from the genus *Pseudomonas* are capable of suppressing a range of plant diseases due to their ability to biosynthesize antimicrobial metabolites. Antibiotics, cyclic lipopeptides (CLPs) with antimicrobial activity, siderophores and hydrogen cyanide are the main secondary metabolites to which the biological control is attributed. Using two strains of the species *P. corrugata* and *P. mediterranea* impaired in CLP production we observed that their antagonistic activity was also the result of the production of other substances such as diffusible siderophores, an additional unknown antimicrobial substance and volatile compounds (VOCs). Putative genes encoding for the hydrogen cyanide (HCN) synthase have been found during genome annotation of *P. corrugata* CFBP 5454. HCN is a VOC produced by many antagonistic *Pseudomonas* species and it was shown that three contiguous structural genes, *hcnABC* operon, encoding together a membrane-bound HCN synthase complex, are sufficient for cyanogenesis. Sequence analysis revealed that the genetic organization of this locus is high similar to other *Pseudomonas* HCN synthase cluster. The putative *hcnA* and *hcnC* genes were insertionally inactivated by using the suicide vector pKNOCK-Km into *P. corrugata* CFBP 5454. Genomic mutants were characterized and the role of this compound in biocontrol activity was investigated. Test paper (Cyantesmo) for the detection of HCN production showed that in *hcnA*- and *hcnC*- mutants metabolite production in completely abolished. *In vitro* experiments against the phytopathogenic fungus *Botrytis cinerea* also showed that HCN production is mainly involved in conidia germination inhibition.

Keywords: *Pseudomonas corrugata*, *P. mediterranea*, secondary metabolites, antimicrobial activity, hydrogen cyanide.

5.2. Introduction

P. corrugata (Roberts and Scarlett) emend. Sutra *et al* . (1997) and *P. mediterranea* (Catara *et a.l*, 2002) are two closely related bacteria both isolated as causal agents of tomato pith necrosis worldwide yet they can be clearly distinguished by phenotypic characters, different genotyping methods and by species specific PCR (Catara *et al*., 2000; 2002; Licciardello *et al*., 2011). *P. corrugata* and *P. mediterranea* are *γ-Proteobacteria* (Kersters *et al*., 1996), belonging to the genus *Pseudomonas sensu stricto* which includes species within rRNA similarity group I or the *fluorescens* rRNA branch (De Vos *et al.,* 1985). Although this genus encompasses essentially fluorescent species, these two species as well as *P. stutzeri* are non-fluorescent. In a phylogenetic study *P. corrugata* and *P. mediterranea* were shown to be part of the *P. fluorescens* group that includes more than fifty named species and where many plant commensal strains are included (Mulet *et al*., 2010). In the *P. fluorescens* group are also included many biocontrol strains with significant effects on agricultural productivity (Loper *et al*., 2012). Successful biological control strains have certain characteristics in common: the capacity to colonize plant surfaces, specifically the infection court of target pathogens; and the production of antibiotics toxic to target pathogens or the induction of systemic resistance responses in the plant (Haas and Défago, 2005; Gross and Loper, 2009; Loper *et al*., 2012). A genomic approach to the study of biocontrol *P. fluorescens* strains reveals that they exhibit a diverse spectrum of traits involved in biological control and other multitrophic interactions with plants, microbes, and insects and that distinct strains have distinct sets of genes related to their distinct biology (Loper *et al*., 2012).

An extensive literature reports that *P. corrugata* have been also isolated from non-diseased plants mainly from root environment and agricultural soils (Catara *et al*., 2007). These strains showed an elevated rhizosphere competence and were foreseen as biological control agent and some of them also patented (Catara, 2007). Of course we don't know if some of these strain are actually *P. mediterranea* since the new species was delineated in 2002 (Catara *et al*., 2002). Strains isolated as commensal as well as those isolated as phytopathogens showed *in vitro* antimicrobial activity against a long list of microorganisms (Gram-negative and Gram-positive bacteria, Chromista, yeast, fungi). A role was suggested for the phytotoxic and antimicrobial cationic cyclic lipopeptides corpeptin A and B and for Cormycin A (Emanuele *et al*., 1998; Scaloni *et al*., 2004).

Moreover, although *P. corrugata* didn't produce pyoverdine commonly produced by the 'fluorescent' species, the siderophore mediated competition for iron could be achieved through the siderophore corrugatin (Risse *et al.*, 1998). Analysis of a *P. corrugata* strain NRRL B-30409 identified in chitinase, siderophore, ammonia and lipase the main mechanisms of action in its biocontrol activity (Trivedi *et al.*, 2008).

What is surprising that screening of *P. corrugata* strains to date revealed many conserved phenotypic traits such as pathogenicity on tomato and a common *in vitro* antimicrobial profile (Catara 2007; Cirvilleri *et al.*, 2001). Recently it has also been demonstrated analyzing a wide collection of strains that *P. corrugata* and *P. mediterranea* produce the same Quorum Sensing acyl homoserine lactone signal molecules, tentatively assigned as C_6-AHL, C_6-3-oxo-AHL and C_8-AHL, with C_6-AHL apparently being the most abundantly produced. (Licciardello *et al.,* 2007; cfr.3.4.1.). Using *P. corrugata* CFBP 5454 and *P. mediterranea* CFBP 5447 as species model strains we found that both possess highly homologous N-acyl Homoserine Lactone (AHL) Quorum Sensing (QS) systems, which relies on two proteins, the AHL synthases (PcoI and PmeI) and their cognate receptors (PcoR and PmeR). In both strains downstream of *luxI* a gene encoding a transcriptional regulator, RfiA, belonging to the LuxR family protein but lacking of the AHL binding domain, was identified. The *rfiA* gene is directly linked to QS by co-transcription with either *pcoI* and *pmeI* in the two bacterial species (Licciardello *et al.*, 2007, 2009, cfr.3.4.1.). The knock out mutants of either the *pcoR* and *pmeR* and *rfiA* are impaired in CLP production and showed reduced virulence when inoculated in tomato stems (cfr. 3.4.4.).

With the aim of investigating the biocontrol mechanism of action of *P. corrugata* CFBP 5454 and *P. mediterranea* CFBP 5447 we analyzed their *in vitro* antagonistic activity related to diffusible and volatile metabolites and the role of CLPs using the above cited impaired mutants. *P. corrugata* activity was also evaluated *in vivo* against model pathosystems. The availability of *P. corrugata* CFBP 5454 genome (Catara *et al.,* unpublished) has also allowed to identify and functionally analyze the cluster involved in hydrogen cyanide biosynthesis (*hcnABC*).

5.3. Material and Methods

5.3.1. Bacterial and fungal strains and media

Strains and plasmids used in this study are listed in Table 7 *Pseudomonas corrugata* CFBP 5454, *P. mediterranea* CFBP 5447 strains and their derivative mutant strains in *luxI*, *luxR* and in the *rfiA* transcriptional regulator homologous genes (Licciardello *et al.*, 2009; 2012) were routinely cultured at 28°C in either nutrient agar (Oxoid, Milan, Italy) supplemented with 1% dextrose (NDA), in Luria-Bertani (LB) agar and on King's medium B (KB) (King *et al.*, 1954). Antibiotics were added as required at the following final concentrations: ampicillin, 100 µg mL^{-1}; tetracycline, 40 µg mL^{-1}; gentamicin, 40 µg mL^{-1}; kanamycin, 100 µg mL^{-1}, nitrofurantoin 100 µg mL^{-1}. *Bacillus megaterium* ITM100 and *Rhodotorula pilimanae* ATTC 26423 were used as bioindicator strains for the presence of cyclic lipopeptides, according to a procedure reported by Lavermicocca and associates (1997).

The antimicrobial activity of the two *Pseudomonas* strains was evaluated against phytopathogenic Gram-positive and -negative bacteria and fungi. The target organisms used in this study are: *Clavibacter michiganensis* subsp. *michiganensis* PVCT 156.1.1; *P. syringae* pv. *tomato* PVCT 28.3.1; *Xanthomonas campestris* pv. *campestris* PVCT 62.4; causal agents of tomato bacterial canker of tomato, and of bacterial speck, and of black rot of brassicacae respectively; they were routinely grown at 27 °C on NDA and KB. The fungal cultures of *Aspergillus niger* and *Penicillum digitatum* PVCT235 agents of postharvest rots; *Botrytis cinerea* causal agent of grey mold in pre- and postharvest; *Fusarium oxysporum* f.sp. *lycopersici* (Saitama ly1) causal agent of Fusarium tomato wilt; *Phoma tracheiphila* PVCT27 causal agent of Mal secco on citrus trees were maintained on Potato Dextrose Agar (PDA).

5.3.2. *In vitro* activity of cell-free bacterial culture broth

P. corrugata and *P. mediterranea* wild type and mutant strain culture filtrates were prepared according to Licciardello and associates to assess CLP production (2009). Bacterial strains were grown in IMM (Surico *et al.*, 1988) at 28 °C for 4 days. After centrifugation (9000 × *g*, 20 min), the supernatant was passed through a 0.22-µm

Millipore filter (Millipore, Billerica, MA, U.S.A.) to obtain cell-free culture filtrates. Aliquots of samples were 10X concentrated for CLP bioassay by using the Vacufuge concentrator 5301 (Eppendorf, Milan, Italy).

Antimicrobial activity was assessed by well-diffusion assay in plates containing PDA overlaid with 6 ml of PDA added with 1 ml of the CLP sensitive strains *R. pilimanae* ATTC26432 and *B. megaterium* ITM100 (approximately 10^6 CFU ml^{-1}). Wells, each 7 mm in diameter, were made in the overlay using a cork borer, and 30 µl of 10X culture filtrates were spotted onto each well. The plates were incubated up to 4 days at 27°C, after which they were examined for clear inhibition zones around the well. All tests were carried out twice in triplicate each time.

5.3.3. *In vitro* activity of *Pseudomonas* strains

Antimicrobial activity was assessed on PDA for detecting antimicrobial CLP production (Bultreys & Gheysen, 1999), and on KB medium for siderophore-mediated competition for iron (King *et al,* 1954). Drops of 10 µl of cell suspension in sterile distilled water of all the *Pseudomonas* strains (10^8 CFU mL^{-1}) were spotted on agar plates, uniformly sprayed with a 10^8 CFU mL^{-1} cell suspension of *C. michiganensis* subsp. *michiganensis* PVCT 156.1.1; *P. syringae* pv. *tomato* PVCT28.3.1 and *X. campestris* pv. *campestris* PVCT 62.4. The plates were incubated at 28°C and the maximum inhibition zones between the two microorganisms were measured.

In order to evaluate the *Pseudomonas* strains antagonistic effects against fungal pathogens small masses from fresh bacterial cultures grown on NDA were transferred onto a Petri dish (1 cm from the edge) containing PDA. Four millimeter mycelia discs from a well grown lawn of the fungal pathogens were cut off by a cork borer and placed in the center of the Petri dish. For each test a control plate was also maintained by adding only test fungal culture. Plates were incubated at 25°C and the antifungal activity was expressed as percentage of growth inhibition (PGI) and calculated according to the formula of Vincent (1947): PGI(%) = 100·(GC-GT)/GC; where GC represent the average diameter of fungal mycelium in PDA (control) and GT represents the average diameter of fungal mycelium on the treated PDA dish contain the antagonistic bacteria. All strains were evaluated in three independent replicates.

Table 7 - Bacterial strains, plasmids and primers used in this study

Strains	Genotype/relevant characteristics	Reference or source
P. corrugata		
CFBP 5454	Wild type, source of *hcnA* and *hcnC*	CFBP[a]
GL1	*pcoI*362::Tn5, Km^r	Licciardello *et al.,* 2007
GL2	*pcoR*76::Tn5, Km^r	Licciardello *et al.,* 2007
GLRFIA	*rfiA*:: pKnock, Km^r	Licciardello *et al.,* 2009
GL1C	GL1 complemented with the cosmid pLC3.34, Tc^r, Km^r	Licciardello *et al.,* 2007
GL2C	GL2 complemented with the cosmid pLC3.34, Tc^r, Km^r	Licciardello *et al.,* 2007
GLRFIAC	GLRFIA complemented with pBBR-*rfiA*, Gm^r,Km^r	Licciardello *et al.,* 2009
PCOHCNA	*hcnA*:: pKnock, Km^r	This study
PCOHCNC	*hcnC*:: pKnock, Km^r	This study
P. mediterranea		
CFBP 5447	Wild type	CFBP[a]
PSMER	*pmeR*:: pKnock, Km^r	Licciardello *et al.,* 2012
PSMEI	*pmeI*:: pKnock, Km^r	Licciardello *et al.,* 2012
PSRFIA	*rfiA*:: pKnock, Km^r	Licciardello *et al.,* 2012
PSMERC	PSMER complemented with the cosmid pLC13.44, Tc^r, Km^r	Licciardello *et al.,* 2012
PSMEIC	PSMEI complemented with the cosmid pLC13.44, Tc^r, Km^r	Licciardello *et al.,* 2012
PSRFIAC	PSRFIA complemented with pBBR-*rfiA*, Gm^r,Km^r	Licciardello *et al.,* 2012
E. coli		
DH5α	F2 f80d*lacZ*D*M15* D(*lacZYA-argF*)*U169 endA1 recA1 hsdR17 deoR gyrA96 thi-1 relA1 supE44*	Sambrook *et al.,* 1989
CC118 λ*pir*	Δ(*ara, leu*)*7697 araD139* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB* (Rf^r) *argE*(Am) *recA1* λ*pir*	Herrero *et al.,* 1990
Plasmid		
pGEM-T	Cloning vector, Amp^r	Promega
pRK2013	Km^r Tra^+ Mob^+ ColE1 replicon	Figurski e Helinski, 1979
pKNOCK-Km^r	Mobilizable suicide vector, Km^r	Alexeyev, 1999
pGEhcnA	*hcnA* internal fragment cloned in pGEM-T	This study
pGEhcnC	*hcnC* internal fragment cloned in pGEM-T	This study
pKM-hcnA	pKNOCK containing an internal fragment of *P. corrugata* CFBP 5454 *hcnA* gene	This study
pKM-hcnC	pKNOCK containing an internal fragment of *P. corrugata* CFBP 5454 *hcnC* gene	This study
Oligonicleotides	**Sequence**	
HCNA-kn fw	5' - AGGATCCAGCCATTAACCAG 3'	This study
HCNA-kn rev	5' – AGGTACCAGGCAGCATTGGCAG-3'	This study
HCNC-kn fw	5' – ATCTAGAATGGTGTCGACC-3'	This study
HCNC-kn rev	5'- AGGTACCGATGAGGATTTC – 3'	This study

[a]CFBP, Collection Francaise de Bacteries Phytopathogenes, Angers, France.

To study the influence of volatile compounds by *Pseudomonas* for antifungal activity a four millimeter mycelia plug of *B. cinerea* and *F. oxysporum* f.sp*. lycopersici* was placed onto PDA plates and investigated by a dual plates assay in which the other plates were inoculated with the bacterial strain. Effect on *B. cinerea* conidial germination was

evaluated spreading 100 µl of conidial suspension $1{\cdot}10^6$ conidia ml^{-1} on PDA plates. In separate PDA dish 100 µl of bacterial suspensions containing $2{\cdot}10^8$ CFU ml^{-1} were spread and then the plate placed over the plate with the fungus, avoiding direct contact between the two, sharing only the air. The two compartment plates were sealed with three layers of parafilm to make a double-dish layer and were incubated at 25°C. The production of volatile compounds was then determined based on inhibition of the PGI of the fungal radial growth or the number of germinated conidia. A conidium was considered as germinated when the germ tube was visible.

5.3.4. HCN production

Hydrogen cyanide (HCN) production was determined qualitatively using Cyantesmo paper (Machery-Nagel GmbH & Co., Düren, Germany). Cyantesmo strips were placed on the lid of LA plates streaked with the *Pseudomonas* strain to test and sealed with three layers of parafilm. Plates were incubated at 28°C for 24 h. HCN production was evaluated by the color change of the strip from green pale to blue.

5.3.5. Identification of HCN biosynthetic cluster and construction of mutants

In order to map the genes involved in putative antimicrobial secondary metabolite production in the *P. corrugata* CFBP 5454 genome, the strain *P. protegens* Pf-5 was used as baits in sequential blastn, blastx and blastp searches. In CFBP 5454 genome only the putative nucleotide sequence of the *hcnABC* cluster was recovered (*P. protegens* Pf-5 *hcnABC* genes: YP_259684/5/6).

Restriction enzyme digestions, DNA fragment isolation from low-melting-point agarose gels, ligation, transformation and agarose gel electrophoresis were performed according to standard procedures as described by Sambrook *et al* (1989). Chromosomal DNA of *P. corrugata* CFBP 5454 was isolated using the Gentra Puregene Cell Kit (Qiagen); plasmids were purified using the BIO BASIC EZ-10 Spin column Plasmid DNA kit. Triparental matings from *E. coli* to *P. corrugata* were carried out with the helper strain *E. coli* DH5α (pRK2013) (Figuraski and Helinski, 1979).

The central part of *hcnA* and *hcnC* genes were amplified by PCR as a 182-bp and 296-bp fragments, respectively, using the primers HCNAkn-fw and HCNAkn-rev, HCNCkn-fw and HCNCkn-rev (Table 7). These fragments were first cloned into pGEM-T (Promega)

according to the manufacturer's instructions, and then subcloned by *BamH*I/*Kpn*I and *Xba*I/*Kpn*I digestions, respectively, into pKNOCK-Km suicide vector (Alexeyev, 1999), generating PKHCNA and PKHCNC respectively. These latter plasmids were transferred into *P. corrugata* CFBP 5454 by triparental mating, generating PCOHCNA and PCOHCNC. Transformants were selected on LB agar plates supplemented with kanamycin (100 µg ml^{-1}), ampicillin (100 µg ml^{-1}) and nitrofurantoin (100 µg ml^{-1}) and confirmed by Southern Blot analysis.

5.3.6. Growth chamber bioassays

In vivo biocontrol activity of *Pseudomonas* strains was tested for the capacity to control *Penicillum digitatum* and *Botrytis cinerea*.

Lemons showing no visible wound were surface-sterilized by dipping for 2 min in 2% of sodium hypochlorite, rinsed in sterile tap water and air-dried under a laminar flow cabinet Fruits were wounded with a sterile scalpel on their peel in four points. Twenty fruits per each strain were used. Drops of 30 µl of bacterial suspensions ($2 \cdot 10^9$ CFU ml^{-1}) were pipetted into each wound and allowed to dry. Subsequently, the wounds were inoculated with 10 µl of conidial suspension $1 \cdot 10^6$ conidia ml^{-1} of the fungal pathogen *P. digitatum*. Positive control fruits were treated with *P. digitatum* only, whereas negative control fruits with the antagonistic strains. Fruits were placed on trays in moist chamber at 20°C. The biocontrol activity was evaluated as percentage of infected wounds.

A bacterial suspension ($1 \cdot 10^8$ CFU ml^{-1}) of each *Pseudomonas* strains was sprayed on the surface of leaves of tomato and zucchini plants and allowed to dry for approximately 2 h. Subsequently, after drying, leaves were inoculated in a thirty total sites with two drops of 10 µl of conidial suspension ($1 \cdot 10^6$ conidia ml^{-1}). Positive control leaves were treated with *B. cinerea* only. Leaves treated with antagonistic strains were also included. Plants were placed in moist chamber at 22°C to optimize condition for disease development. The antagonistic activity was evaluated as percentage of sites showing symptoms of infection.

5.4. Results

5.4.1. *In vitro* antimicrobial activity of *P. corrugata* CFBP 5454 and *P.mediterranea* CFBP 5447

In order to determine the effects of *P. corrugata* CFBP 5454 and *P. mediterranea* CFBP 5447 strains on fungal mycelia and bacterial growth, dual-culture antagonism assays were performed on PDA plates. Five plant pathogenic fungi, *Aspergillus niger, Botrytis cinerea, Phoma tracheiphila, Fusarium oxysporum* f.sp. *lycopersici*, *Penicillum digitatum* were tested. Both *Pseudomonas* strains significantly inhibited the mycelial growth of all the fungi, that showed percentages of growth inhibition (PGI) ranging from 38 to 56% for CFBP 5454 and from 35 to 53% for CFBP 5447 as compared to the control plates containing only the target fungus (Fig. 26; 29A). Both bacteria showed the highest antagonistic effect towards *A. niger, B, cinerea* and *P. digitatum* (Fig. 26; 29A). The inhibitory activity was evidenced by the limited growth of fungal mycelia in the inhibition zone in front of the bacterial spot .

In tests against phytopathogenic bacteria both *P. corrugata* CFBP 5454 and *P. mediterranea* CFBP 5447 showed the highest antimicrobial activity against the Gram-positive phytopathogenic bacterium *Clavibacter michiganensis* subsp. *michiganensis* with inhibition halos of approximately 9 mm. Both strains inhibited, although to a lesser extent, the growth of *P. syringae* pv. *tomato* but only *P. corrugata* was able to antagonize *X. campestris* pv. *campestris* (Fig. 27).

In order to evaluate the role of CLPs in the antagonistic activity of the two *Pseudomonas* strains we tested both bacterial cells and cell-free culture broth produced in CLP inducing conditions (Surico *et al.,* 1988) against the two CLP bioindicator microorganisms, the yeast *Rhodotorula pilimanae* and the Gram-positive bacterium *Bacillus megaterium*, according to Lavermicocca *et al.,* (1997). Both *P. corrugata* CFBP 5454 and *P. mediterranea* CFBP 5447 cells and cell free culture filtrates showed antimicrobial activity against the two CLP bioindicators producing large inhibition halos (Table 8).

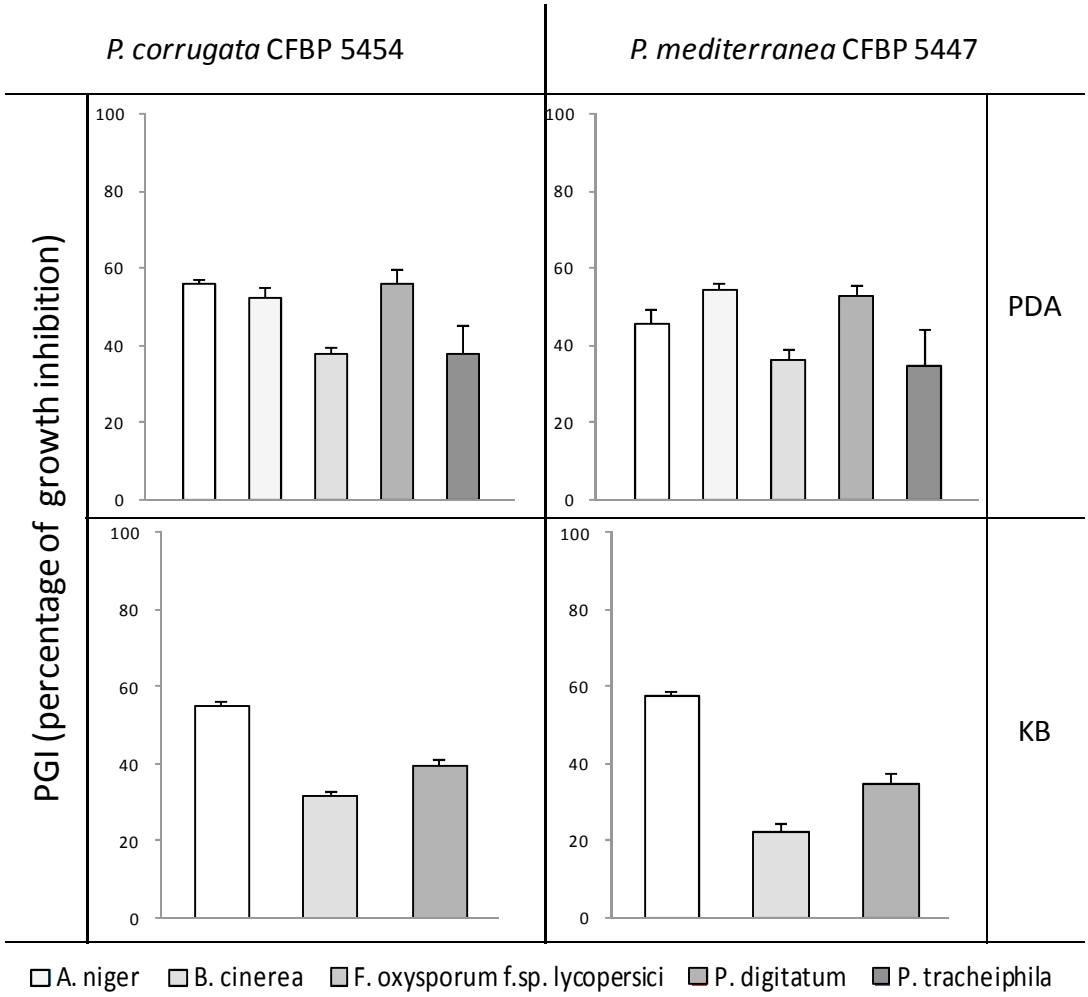


Fig. 26 - Antifungal activity of *P. corrugata* CFBP 5454 and *P. mediterranea* CFBP 5447, against phytopatogenic fungi on PDA and KB for diffusible compounds and siderophore evaluation respectively. Bars represents the percentage of fungal growth inhibition. Error bars represent standard deviations.

The bacterial strains were also tested in dual cultures against the above cited fungi on KB agar plates to determine if siderophores play a role in antagonism activity. No significant differences were observed, among the two *Pseudomonas* strains, which showed PGI of approximately of 58%, 40%, and 30% against *A. niger*, *P. digitatum* and *F. oxysporum* f.sp. *lycopersici,* respectively (Fig. 26; Fig. 29B). *F. oxysporum* f.sp*. lycopersici* showed a PGI of approximately 30% (CFBP 5454) and 20% (CFBP 5447) when compared to the control plate, but on the 15th day of incubation, the mycelium had covered almost completely the bacterial spots. *B. cinerea* and *P. tracheiphila* mycelia were not able to

grown on KB. *P. corrugata* and *P. mediterranea* on KB agar showed activity only against *C. michiganensis* subsp*. michiganensis* with inhibition halos of approximately 9 mm whereas no activity was shown against *P. syringae* pv. *tomato* and *X. campestris* pv. *campestris* (Fig. 27).

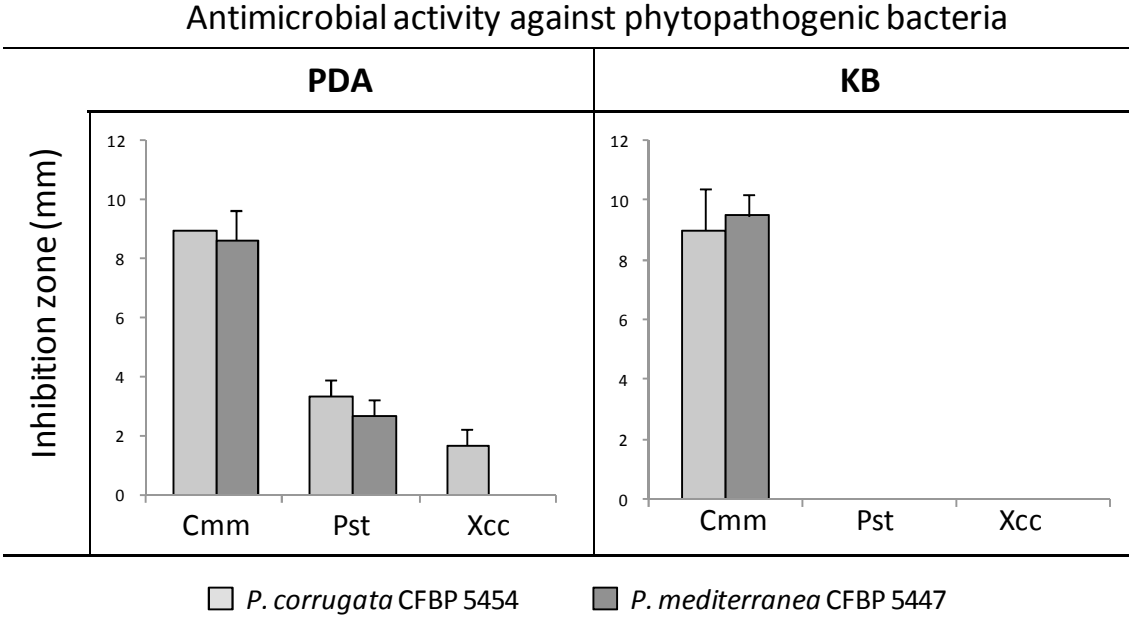


Fig. 27 - Antimicrobial activity of *P. corrugata* CFBP 5454 and *P. mediterranea* CFBP 5447, against phytopathogenic bacteria: *Clavibacter michiganenis* subsp. *michiganensis* (Cmm); P. *syringae* pv. tomato (Pst) and *X. campestris* pv. *campestris* (Xcc), on PDA and KB. Bars indicated the values of the inhibition zones (mm). Results are representative of two different experiments with three replicates for each.

P. corrugata and *P. mediterranea* were challenged each other both on PDA and KB agar plates and no antagonistic activity was observed.

The potential antifungal activity of volatile compounds produced by *P. corrugata* and *P. mediterranea* was tested against *B. cinerea* and *F. oxysporum* f.sp. *lycopersici*. A PGI of 60% was observed when *B. cinerea* was exposed to either bacterial cultures. Control plates showed significant mycelial growth and typical grey pigmentation due to fungal sporulation, in contrast with the mycelium which appeared white in presence of the two *Pseudomonas* strains. Microscopic analysis confirmed the absence of sporulating structures in the white mycelium. *F. oxysporum* f.sp. *lycopersici* showed less inhibition,

probably due to the fast fungal growth, although a change of color was observed in presence of *P. corrugata* (Fig. 28).

Strains	***P. corrugata* CFBP 5454**					***P. mediterranea* CFBP 5447**				
	CLP *	CF		BC		CLP *	CF		BC	
		Rp	*Bm*	*Rp*	*Bm*		*Rp*	*Bm*	*Rp*	*Bm*
WT	CM-A, CPs	6 ± 0,5	5 ± 0,45	7 ± 0,8	7,33 ± 0,6	CM-A, CPs	6 ± 0,5	4 ± 0,5	8 ± 0,8	8 ± 0,7
LuxI-	CM-A, CPs	0	0	7,33 ± 0,5	7,33 ± 0,6	-	0	0	7,33 ± 0,6	6 ± 0,8
LuxR-	-	0	0	7,33 ± 0,5	7,33 ± 0,6	-	0	0	7,33 ± 0,6	6 ± 0,8
RfiA-	-	0	0	0	8 ± 1	-	0	0	0	4,7± 0,8

* From MALDI-TOF data for CLP production in *P. corrugata* CFBP 5454, *P. mediterranea* CFBP 5447 and derivative QS and RfiA mutant strains (Licciardello *et al.*, 2012).

Table 8 - Antimicrobial activity of cell free culture filtrates (10×) (CF) and bacterial cells (BC) recovered on inducing conditions against *Rhodotorula pilimanae* and *Bacillus megaterium* indicator microorganisms on PDA medium. Values of the inhibiton zones (mm) are the means of three replications. Results presented are representative of three different experiments.

5.4.2. *In vitro* antimicrobial activity of *Pseudomonas* mutants impaired in CLP production

P. corrugata and *P. mediterranea* produce in culture the antimicrobial, phytotoxic and surfactant cyclic lipopeptides (CLP) corpeptins and cormycin (Emanuele *et al.,* 1998; Scaloni *et al.,* 2004; cfr. 3.4.4.). We previously demonstrated that *P. corrugata* CFBP5454 and *P. mediterranea* CFBP5447 knock out mutants of QS *luxR* homologs *pcoR* and *pmeR*, respectively and of the transcriptional regulator *rfiA* were impaired in CLP production. Differences were instead observed in the LuxI homologous mutants, because of the *pcoI* mutant, although at a reduced level, still produce both cormycin and corpeptins (Licciardello *et al.,* 2009,cfr 3.4.4.).

Thus, to focus on the role of CLPs and to investigate the presence of other antimicrobial compounds, the insertional mutants of the *luxI*, *luxR* and *rfiA* (called LuxR-RfiA -and LuxI-) were tested in dual-culture antagonism assays on PDA and KB plates.

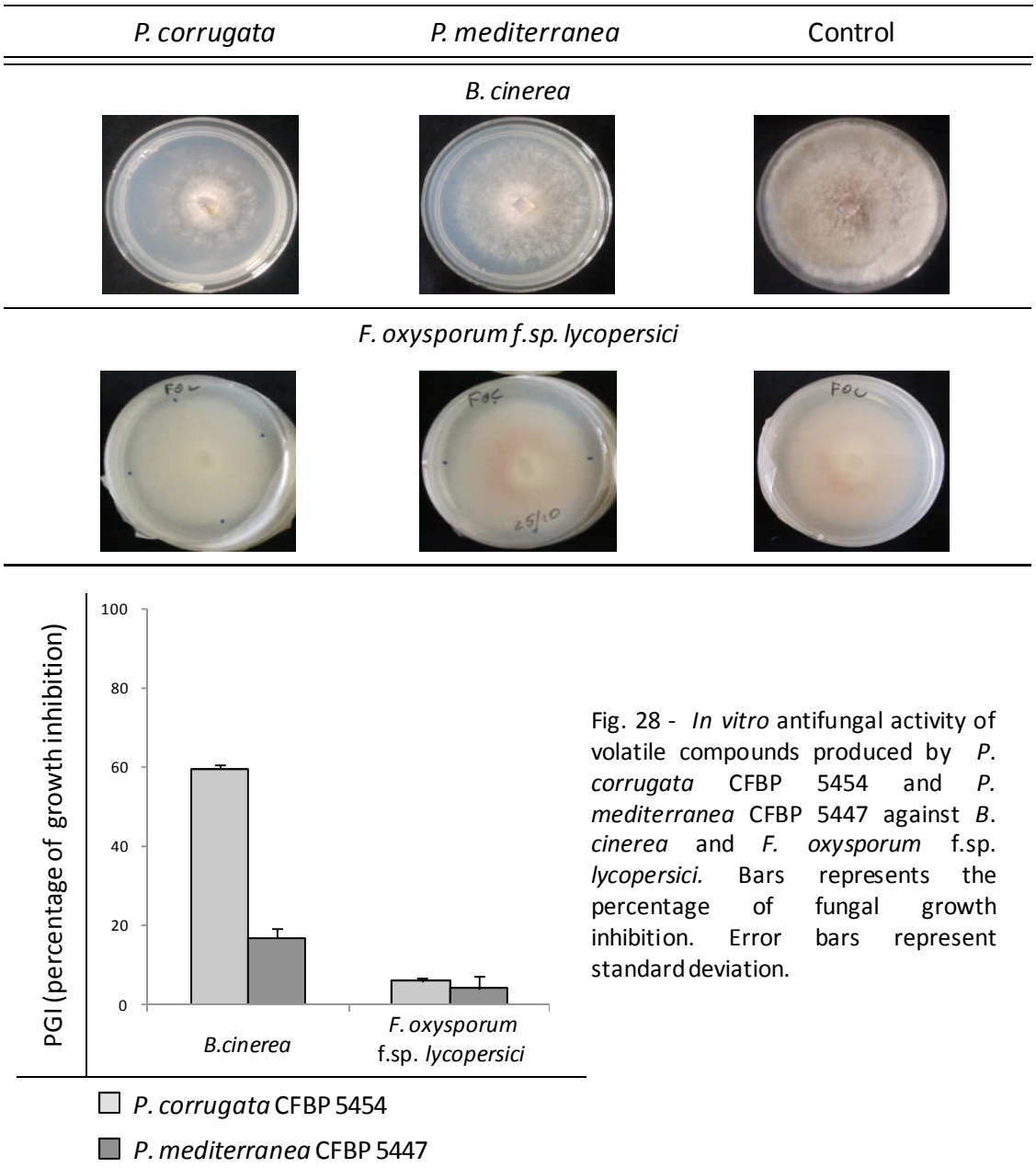
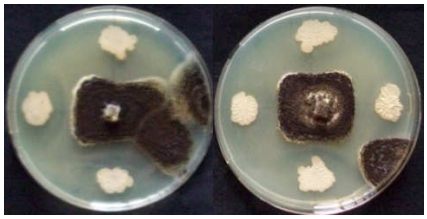
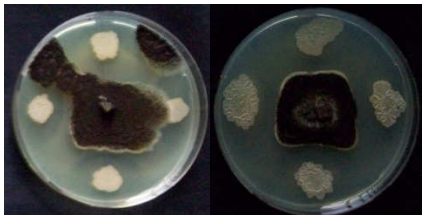
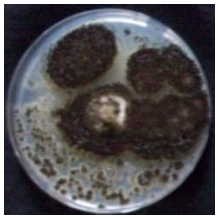
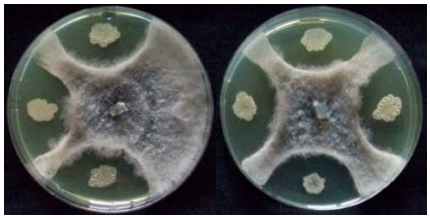
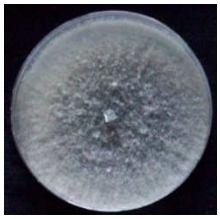
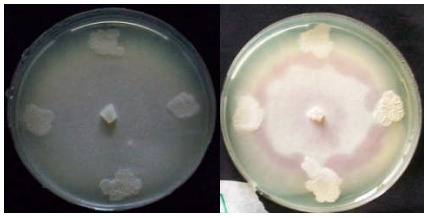
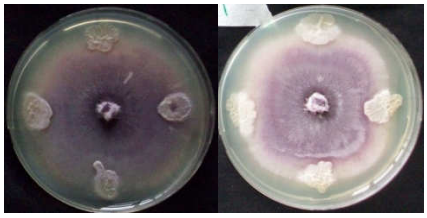
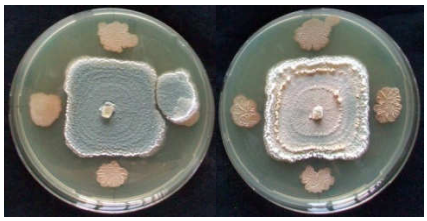
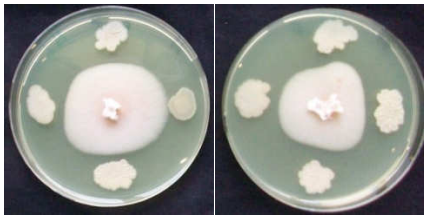


Fig. 28 - *In vitro* antifungal activity of volatile compounds produced by *P. corrugata* CFBP 5454 and *P. mediterranea* CFBP 5447 against *B. cinerea* and *F. oxysporum* f.sp. *lycopersici.* Bars represents the percentage of fungal growth inhibition. Error bars represent standard deviation.

Antifungal activity on PDA against *A. niger, B. cinerea*, *P. digitatum* and *P. tracheiphila* was significantly reduced in RfiA- mutant strains 7 days after incubation when compared to parental strains of both species. Fifteen days after incubations *A. niger, B. cinerea*, *P. digitatum* mycelia overgrowth RfiA- bacterial spots. No significant differences were instead observed between LuxR- and LuxI- mutants and their respective parental strains. The inhibitory activity was restored in the RfiA- mutant strains of both species

P. corrugata		*P. mediterranea*		Control
Mutant strains	Complemented strains	Mutant strains	Complemented strains	
A. niger				
				
B. cinerea				
				
F. oxysporum f.sp. lycopersici				
				
P. digitatum				
				
P. tracheiphila				
				

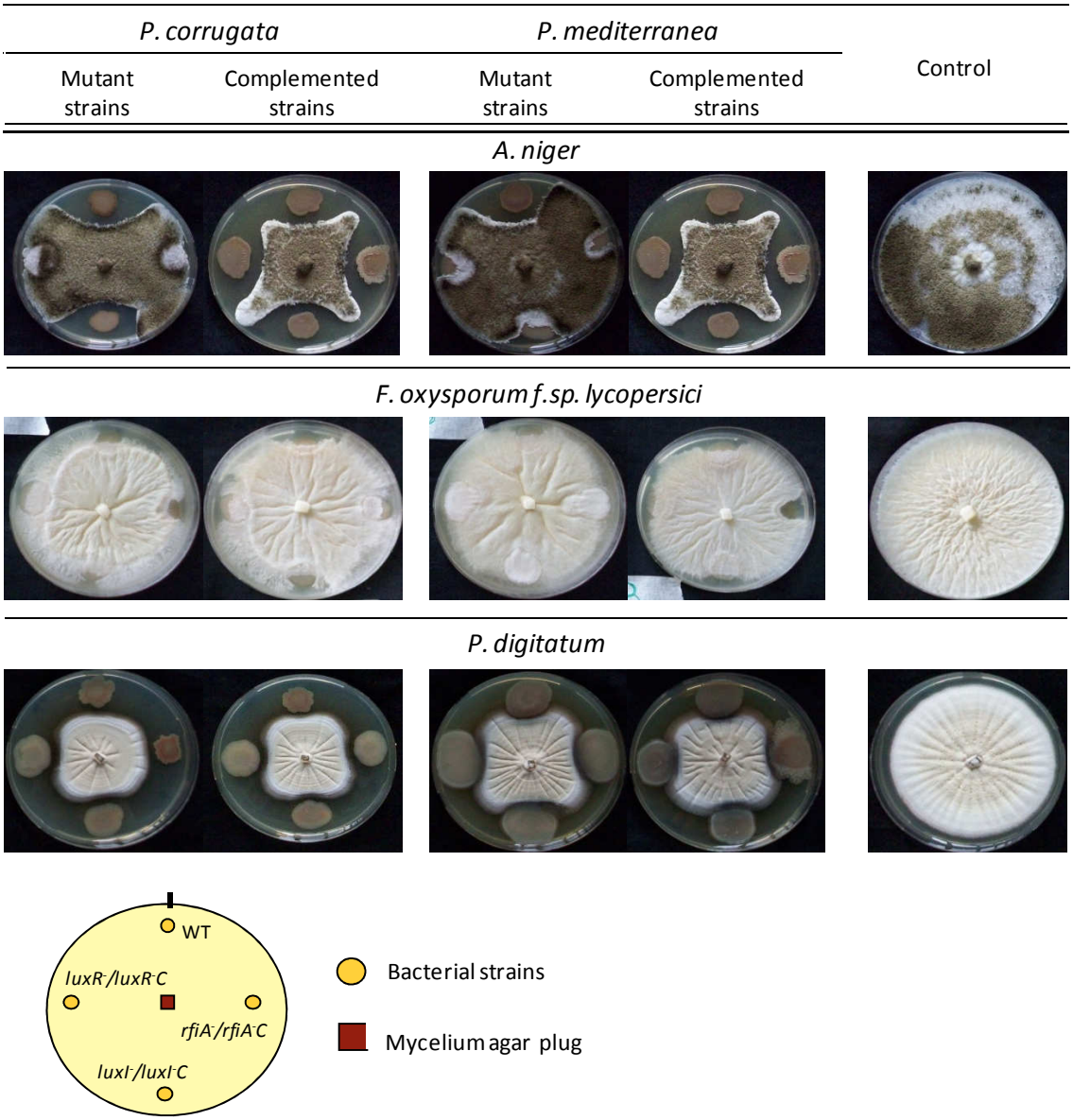


Fig. 29 - Antifungal activity of *P. corrugata* CFPB 5454 and *P. mediterranea* CFPB 5447 and derivative *luxR*$^{-}$ (GL2, PSMER), *luxI*$^{-}$ (GL1, PSMEI), *rfiA*$^{-}$ (GLRFIA, PSRFIA) mutant and complemented strains on PDA (A) and KB (B) for diffusible compounds and siderophore evaluation respectively. The scheme above indicate the disposition of the tested strains in the plates.

complemented *in trans* with the pBBR-RfiA construct containing the homologue *rfiA* gene of *P. corrugata* CFBP 5454 (Licciardello *et al.,* 2009). No inhibition of *F. oxysporum f. sp. lycopersici* growth was determined by LuxI-, LuxR- and RfiA- mutant strains, thus showing a behavior similar to the parental strains.

Further antimicrobial assay were carried out on bacterial cultures and cell free culture filtrates toward the CLP biosensors (see above). The antimicrobial activity was completely abolished in the RfiA- mutants of both species against *R. pilimanae*, whereas it was still maintained against *B. megaterium,* as well as in the LuxI- and LuxR- mutant strain. Reduced inhibition halos were instead exhibited by *P. mediterranea* LuxR- and RfiA- mutant strains against *B. megaterium* compared the parental strain.

P. corrugata and *P. mediterranea* LuxR- and RfiA- mutant strains antifungal activity on KB agar plates was drastically reduced in both mutants toward *A. niger* and on the 15th day mutant bacteria spots were completely covered by the fungal mycelium. Differences were instead observed between the LuxI- mutants, because of the one of *P. corrugata* maintained its inhibitory activity against *A.niger.* No significant differences were instead observed in LuxI-, LuxR- and RfiA- mutant strain inhibitory activity against *F. oxysporum* f.sp. *lycopersici* and *P. digitatum* when compared to their parent strains.

5.4.3. *In vivo* antagonism of *P. corrugata* CFBP 5454

P. corrugata strain CFBP 5454 and its derivative mutants impaired in CLP production (LuxR- and RfiA-) were evaluated for their biocontrol activity against two phytopathogenic fungi in different hosts systems: *P. digitatum* on lemon fruits and *B. cinerea* on tomato and zucchini leaves.

Lemon fruits treated only with the *P. digitatum* (positive control) starting from the 5th days after inoculation, exhibited initial soft rot and on the 7th day the presence of white mycelium and subsequently sporulation was observed with a disease index of approximately 90% (Fig. 30A). In contrast no reaction was observed in most of the fruits treated only with *Pseudomonas* strains (negative control). Disease incidence in lemon fruits treated with *P. corrugata* wild type strain was of average of 20% and LuxI- LuxR- and RfiA- mutants did not displayed a significant reduced biocontrol activity (Fig. 30B).

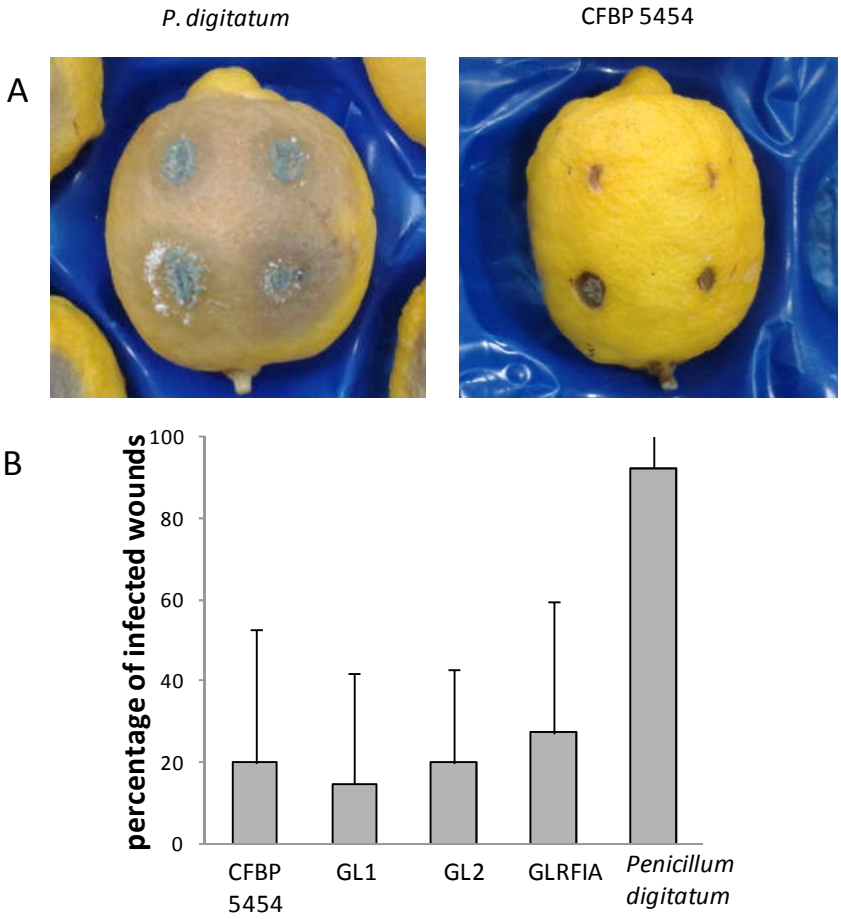


Fig. 30 - (A) *P. digitatum* on lemon fruits. On the left fruit inoculated only with *P. digitatum* ; on the right fruit inoculated with *P. digitatum* and *P corrugata*. (B) Percentage of infected wounds with *P. digitatum* in fruits treated with *P. corrugata* CFBP 5454, GL1, GL2 and RfiA mutant strains . Error bars represent standard deviation.

The inoculation of zucchini and tomato leaves with *B. cinerea* resulted in a disease incidence of 100% and 94% respectively and the inoculated sites showed necrotic lesions surrounded by a chlorotic halo. No lesions were present on leaves inoculated only with the bacterial strains (Fig. 31A). On zucchini and tomato leaves treated with *P. corrugata* CFBP 5454 only the 20% and 18% of *B. cinerea* positive infections were recorded respectively (Fig. 31B). On tomato LuxR- and RfiA- CLP impaired mutants showed a reduced activity, with a 44% and 33% incidence of disease respectively, compared to the parental strain biocontrol activity. No relevant differences were instead observed on zucchini leaves trated with the WT strain and derivative mutants (Fig. 31B).

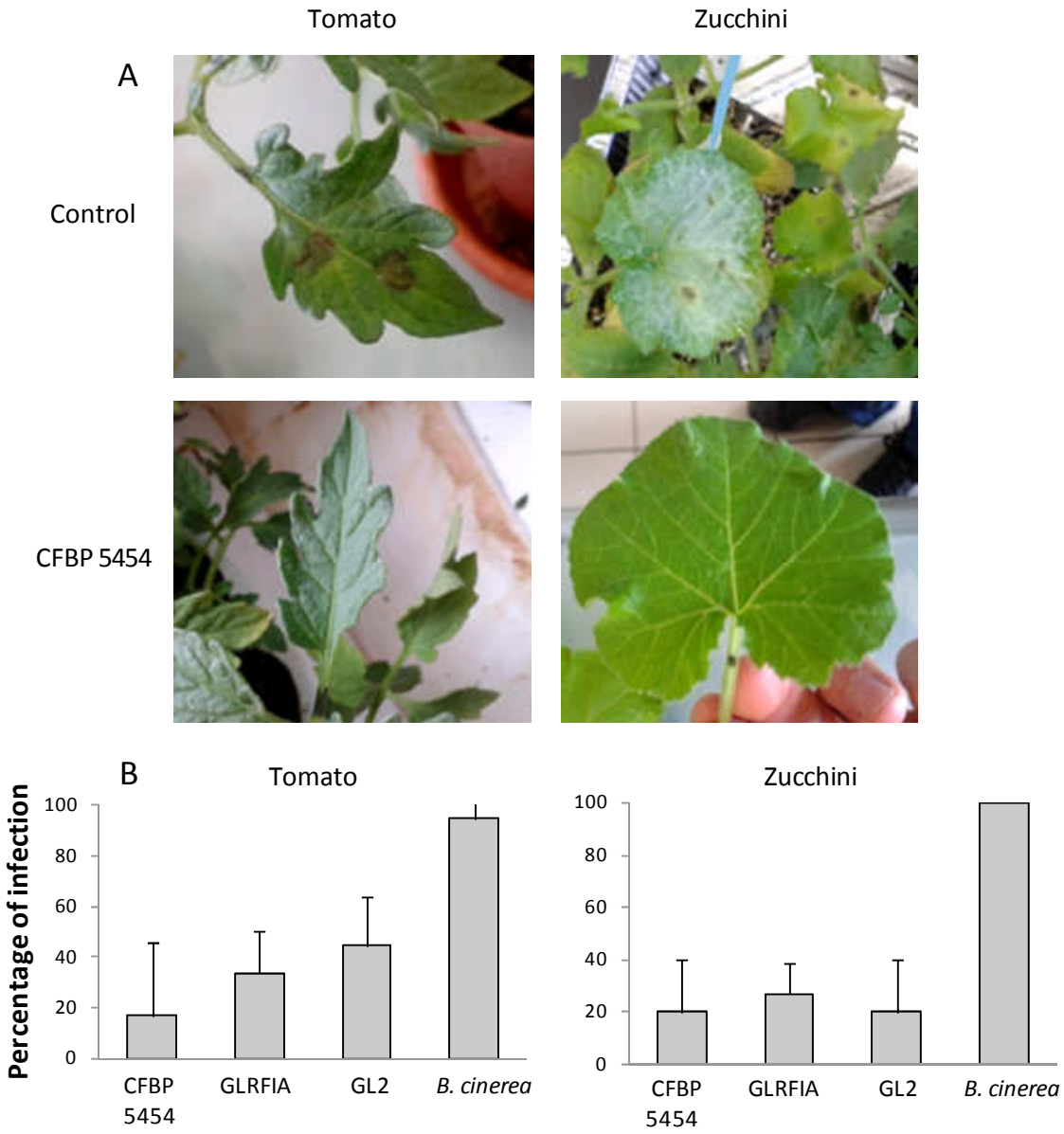


Fig. 31 - (A) Antifungal activity of *P. corrugata* against *B. cinerea* on tomato and zucchini leaves. (B) Percentage of infected sites with *B. cinerea* in leaves treated with *P. corrugata* CFBP 5454, GLRFIA and GL2 mutant strains. Error bars represent standard deviation

5.4. 4. Role of cyanide production in *P. corrugata* CFBP 5454

The production of volatiles is a widespread feature of biocontrol pseudomonads and a cyanide biosynthesic locus was found in the genomes of a number of *P. fluorescens* strains such as Pf-5, 30-84, O6, In5 (Ramette *et al.,* 2003; Loper *et al.,* 2012; Michelsen and

Stougaard, 2012). When *P. corrugata* CFBP 5454 and *P. mediterranea* CFBP 5447 agar cultures were exposed to Cyantesmo test strips a color change from light green to deep blue was observed in response to the detection of liberated cyanide (Machery-Nagel GmbH & Co., Düren, Germany, manufacturer's instructions). The draft genome sequence of CFBP 5454 was just available to our research group during the course of these trials (genbank accession number ATKI00000000) thus we though to mine the genome for genes involved in cyanide production. The *P. protegens* Pf-5 *hcnABC* sequence, was used as baits for the *in silico* data mining of the *P. corrugata* CFBP 5454 genome.

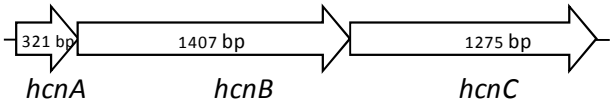
This procedure identified an HCN synthase locus within a 3.003-bp DNA region. Three contiguous open reading frames (ORFs), designated *hcnA hcnB* and *hcnC* were identified. The stop codon (CCA) of *hcnA* overlapped the start coding region of *hcnB* by 1 bp and, similarly, the stop codon (GAC) of *hcnB* overlapped the start coding region of *pcoC* by 5 bp. Their structural organization suggests that they form an operon (Fig. 32A).

The *hcnA*, *-B*, and *-C* genes are 321, 1407, and 1275 bp in size, respectively, and composition analysis revealed that the G+C contents range from 64 to 66%. At the amino acid sequence level, HcnA displayed 85% sequence identity (88% homology) with a putative HcnA in *P. fluorescens* F113 (YP_005207773.1). Conserved domain analysis revealed the presence of a highly conserved cluster of four cysteine residues in the in HcnA protein which resembles a similar sequence motif in ferredoxins and may interact with a [2Fe-2S] center. These kind of protein plays an important role in the electron transfer in redox reactions (Fig. 32B; Fig. 32C). The analysis of the putative HcnB and HcnC protein showed 91% and 94% sequence identity (95% and 96% similarity) respectively to putative HcnB and HcnC protein in *P. fluorescens* F113 (YP_005207774.1, YP_005207775.1). Conserved domains search revealed that HcnB and HcnC each have a typical NAD(P)- or FAD- binding motif. Membrane Protein Explorer (MPex) output, predicted 1, 5 and 3 transmembrane segment in *P. corrugata* CFBP 5454 HcnABC protein respectively, confirming that HCN synthase is a membrane-bound enzyme. No *lux* box like elements were found in the *hcnABC* promoter sequence (Fig. 32B; Fig. 32C).

P. corrugata CFBP 5454 *hcnA* and *hcnC* were insertionally inactivated creating two genomic mutants designated PCOHCNA and PCOHCNC. The two mutants no longer produced HCN as detected by using the Cyantesmo kit (Fig. 33C). To evaluate if the QS or the RfiA regulator have a role in HCN production we also tested cultures of GL1, GL2,

GLRFIA and PSMER, PSMEI PSRFIA mutant strains and all of them still produce HCN (data not shown).

A



B

Protein	% identity	% homology		ID protein
HcnA				
protein HcnA	85	88	*P. fluorescens* F113	YP_005207773.1
hydrogen cyanide synthase HcnA	84	88	*P. brassicacearum* NFM421	YP_004354685.1
(2Fe-2S)-binding protein	77	86	*Pseudomonas* sp. GM30	WP_007965626.1
HcnB				
protein HcnB	91	95	*P. fluorescens* F113	YP_005207774.1
hydrogen cyanide synthase HcnB	91	95	*P. brassicacearum* NFM421	YP_004354684.1
(2Fe-2S)-binding protein	84	90	*Pseudomonas* sp. GM79	WP_008070661.1
HcnC				
protein HcnC	94	96	*P. fluorescens* F113	YP_005207775.1
hydrogen cyanide synthase HcnC	94	96	*P. brassicacearum* NFM421	YP_004354683.1
glycine/D-amino acid oxidase, deaminating	90	93	*Pseudomonas* sp. GM79	WP_008070658.1

C

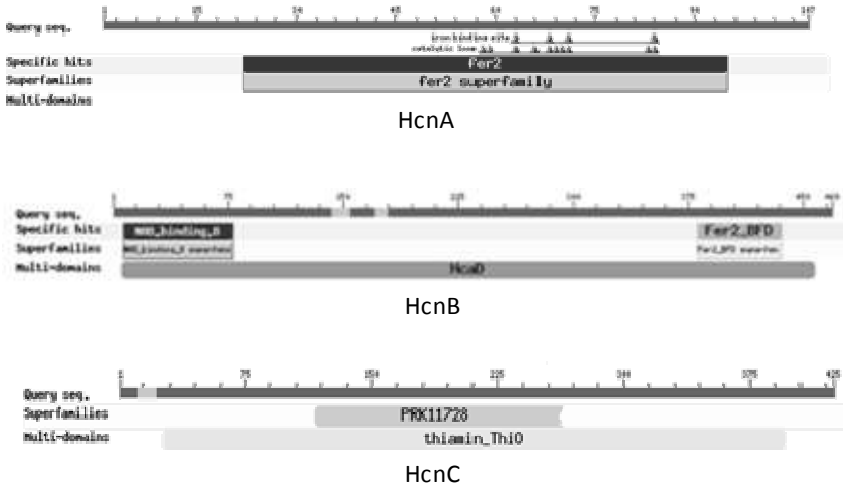


Fig. 32 - (A) Gene map of a 3-Kb DNA region of *Pseudomonas corrugata* CFBP 5454. Genes *hcnA, hcnB* and *hcnC* form the *hcnABC* operon, encoding for the HCN synthase. (B) Blastp comparison between deduced HcnABC protein sequences from *P. corrugata* CFBP 5454 and closest relatives. (C) Graphical summary of similar conserved domain (CD) outputs for HcnA, HcnB and HcnC deduced protein from CD-Search in NCBI interface (www.ncbi.nlm.nih.gov).

WT strain and PCOHCNA and PCOHCNC (*hcnA*- and *hcnC*-, respectively) mutant strains inhibited the *B. cinerea* mycelial growth with PGI values of approximately 44, 25, and 23% respectively in comparison with the control plates (Fig. 33A). Thus HCN production seems involved in fungal growth inhibition. Volatile antagonistic effect of the WT and derivative mutant strains was also evaluated on *B. cinerea* conidial germination. One day after incubation under a binocular light microscope the production of the germ tubes was observed only in the conidia of the control plates. Seven days after incubation control plates were covered by a mycelium layer and abundant new conidia production. Few conidia germinated forming very small colonies in plates challenged with *P. corrugata* WT strain whereas plates challenged with the two HCN impaired mutants showed an intermediate phenotype, i.e. the conidia germinated forming colonies and no new sporulation was observed (Fig. 33C). Two days after incubation, some replicate plates of each treatment were separated from the plates containing the bacteria and singularly incubated at 25°C. In all treatments including the WT strain challenged plates *B. cinerea* conidia were still able to germinate (data not shown).

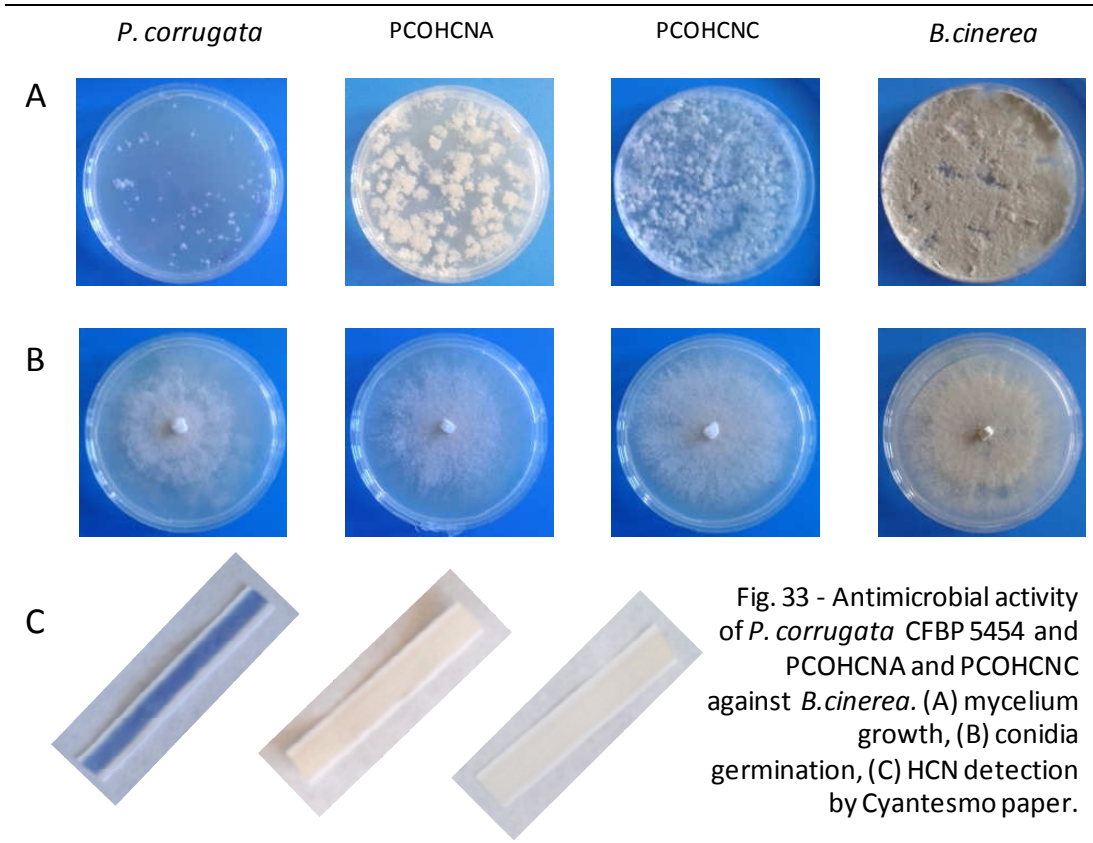


Fig. 33 - Antimicrobial activity of *P. corrugata* CFBP 5454 and PCOHCNA and PCOHCNC against *B.cinerea*. (A) mycelium growth, (B) conidia germination, (C) HCN detection by Cyantesmo paper.

5.5. Discussions

P. corrugata although isolated as a plant pathogen is considered an efficient biocontrol agent in many studies (Catara, 2007). In numerous studies the *in vivo* activity of *P. corrugata* was demonstrated (Catara, 2007). In this study, two strains, *P. corrugata* strain CFBP 5454 and *P. mediterranea* strain CFBP 5447 used as model strains in our laboratories, were investigate to shed some light on their possible mechanism of action and efficiency as biological control agents. Recently the whole genome sequence (WGS) of both strains was obtained and the annotation of the *P. corrugata* strain completed, deposited in genbank (at NCBI, http://www.ncbi.nlm.nih.gov/) and of imminent publication (Catara *et al.,* 2013).

Our results showed that CFBP 5454 and CFBP 5447 strains produced diffusible compounds which have the capacity to inhibit the *in vitro* growth of plant pathogen fungi and bacteria investigated in this study, with minor quantitative difference between the two strains. The main difference was that the *P. mediterranea* strain was not able to inhibit the growth of *X. campestris* pv. *campestris*.

Antimicrobial activity of *P. corrugata* was thought to be due mainly to the production of the CLPs corpeptins (Emanuele *et al.,* 1998) and cormycin (strain dependant, Scaloni *et al.,* 2004) and to the siderophore corrugatin (Risse *et al.,* 1998). Both mechanisms of action seem involved in our bacterial strains since they are active both on PDA (Bultreys and Gheysen, 1999) and KB agar (Bultreys *et al.,* 2001). The main antagonistic mechanism of CLPs is believed to be pore formation which disrupts the electrical potential across the cell membrane (Bender *et al.,* 1999); thus different antifungal efficiency observed could depend on fungal on their chemical plasma membrane sterol content (Latoud *et al.,* 1990).

The antimicrobial activity observed on PDA against the gram-positive bacterium *Clavibacter michiganensis* subsp. *michiganensis*, was greater than against the Gram-negative *P. syringae* pv. *tomato.* It is possible that the reduced or null activity of diffusible compounds produced by CFBP 5454 and CFBP 5447 strains against Gram-negative bacteria could be ascribed to protective effects of the outer membrane (Nybroe and Sørensen 2004). However, this general observation does not apply to all CLPs; in fact, surfactin by *Bacillus subtilis* strain 6051 was shown to have bactericidal activity against *P. syringae* pv. *tomato* and toolasin I by *P. toolasi* against several gram-negative strains (Bais *et al.,* 2004; Lo Cantore *et al.,* 2006).

We previously demonstrated that *P. corrugata* CFBP 5454 and *P. mediterranea* CFBP 5447 strains produce in culture both corpeptins and the nonapeptide cormycin A (cfr. 3.4.4.). Both CLPs inhibited the *in vitro* growth of the Gram positive bacterium *Bacillus megaterium* and cormycin also exhibited activity against the yeast *Rhodotorula pilimanae* (Emanuele *et al.,* 1998; Scaloni *et al.,* 2004).

We previously observed that the cell-free culture filtrates of the knock out mutant strains in the genes encoding the QS LuxR transcriptional regulators PcoR and PmeR of *P. corrugata* CFBP5454 and *P. mediterranea* CFBP 5447, respectively, and in those encoding the transcriptional regulator RfiA common to both species did not contain corpeptins and cormycin (cfr. 3.4.4.). Thus we used these four mutant strains in antagonistic tests. Tests against CLP bioindicator strains, using the 10X concentrate cell-free culture filtrates, did not detect any more antimicrobial activity of both LuxR- and RfiA- mutants. Nevertheless tests using bacterial cells pointed out that supplementary antimicrobial metabolites are produced by both bacterial strains. In particular the LuxR- mutants although did not produce CLP were still able to inhibit the growth of both *R. pilimanae* and *B. megaterium*, whereas the activity against the yeast cells were impaired in the RfiA- of both bacterial strains. The failure of RfiA- strain to produce substance able to inhibit fungi was also confirmed in the assays against the plant pathogenic *A. niger, B. cinerea, P. digitatum* and *P. tracheiphila* .These evidence suggests that at least other two metabolites one targeted to fungi and regulated by RfiA and another antibacterial metabolite are produce by the two srains. Moreover RfiA- mutants of *P. corrugata* and *P. mediterranea* when inoculated for pathogenicity test in tomato plantlets showed a drastic reduction in virulence with absence of necrosis in the inoculated stems (Licciardello *et al.,* 2009; cfr 3.4.2.). Thus a crucial role of RfiA in interactions with eukaryotes cannot be ruled out as well as the involvement of other secondary metabolites.

In vivo trials showed that *P. corrugata* CFBP 5454 was able to reduce infections of *P. digitatum* on lemon fruits and of *B. cinerea* on tomato and zucchini leaves. The bacterium seems actually to interfere with initial steps of fungal/plant interaction since the main effect relies on the reduction of number of positive infections as demonstrated in the results section. Co-inoculation of the fungi with the two mutants PcoR– and RfiA- determined disease incidence values in the different pathosystems not different to those registered in the fruits/leaves treated with the WT strain thus suggesting the CLP and/or additional antimicrobial substances produced by *P. corrugata* are not essential in its

biocontrol mechanism of action. The involvement of mechanisms related to the induction of plant defense responses cannot be ruled out since other authors demonstrated the ability of *P. corrugata* (strain 13) to reduce the infection of *Pytium aphanidermatum* on cucumber when inoculated in a split root experiment in which the pathogen and the bioinoculant were spatially separated (Paultiz *et al.,* 1992). The inoculation with that strain also led to the accumulation of salicylic acid in treated cucumber plants (Chen *et al.,* 1999). Moreover live bacterial cells and *P. corrugata* culture liquid elicit phytoalexin (medicarpin) biosynthesis in white clover callus and K^+/H^+ exchange in tobacco leaf discs and transient formation of active oxygen species (hydrogen peroxide and superoxide) in clover callus cells (Gustine *et al.,* 1990, 1994, 1995).

The role of CLPs in biocontrol is controversial and dependant on pathosystems analysed; biocontrol agents may produce CLPs but in some cases these molecules are not required for disease suppression (Tran *et al*., 2007; Mazzola *et al*., 2007; Berry *et al*., 2010). A massetolide-deficient mutant of *P. fluorescens* strain SS101 was less effective in suppressing late blight of tomato (Tran *et al*., 2007), but exhibited wild-type biocontrol of *Pythium* mediated root rot of apple and wheat (Mazzola *et al*., 2007). In other trials both the incidence of leaf infection and disease severity were markedly increased in *Pseudomonas* DF41 *lp* mutant (impaired in CLP production) suggesting that this metabolite was essential for DF41 biocontrol of *S. sclerotiorum* stem rot of canola (Berry *et al*., 2010).

Some authors described for *P. corrugata* strains the production of other secondary metabolites or the detection of genes putatively involved in their production and deposited in GenBank such as: inhibitory volatiles (Fernando *et al.,* 2005), hydrogen cyanide (Ramette *et al.,* 2003), ammonia (Trivedi *et al.,* 2008) 2,4-diacetylphluoroglucinol (GenBank nos. AJ515694, AJ515693, AJ515692), pyrrolnitrin (Garbeva *et al.,* 2001), but none of these aspects were further investigated. *P. corrugata* CFBP5454 genome mining by BLAST research using as baits genes encoding a spectrum of traits involved in biological control and identified in the genomes of several sequenced *P. fluorescens* strains (Loper *et al.,* 2012) showed it doesn't have genes involved in the synthesis of antimicrobial compounds like phenazines, pyoluteorin, pyrrolnitrin, or 2,4-diacetylphloroglucinol (data not shown) but has a biosynthetic cluster for the enzyme HCN synthase involved in this volatile compound biosynthesis.

By the fact, our results also specify that *P. corrugata* and *P. mediterranea* produce volatile compounds, which seem to play an important role in fungal growth inhibition. *B. cinerea* mycelia growth was reduced by the presence of both species, yet *P. corrugata* showed the greatest inhibition ability. A role is probably due to Hydrogen cyanide (HCN) production, a poisonous secondary metabolites produced by several *Pseudomonas* species, with antagonistic properties (Voisard, *et al.,* 1989). *P. corrugata* CFBP 5454 and *P. mediterranea* CFBP 5447 either produce HCN as detected by Cyantesmo paper test.

The HCN synthesis gene cluster was identified in CFBP 5454, and DNA sequence analysis showed similarity to known *hcnABC* gene clusters. Nucleotide sequence analysis revealed that these three genes encode for the enzyme which catalyses the formation of HCN and CO_2 from glycine and it is believed to be a membrane-bound flavoprotein (Wissing and Andersen 1981). The *hcnA* and *hcnB* ORFs and the *hcnB* and *hcnC* ORFs of CFBP 5454 were found, as in *P. fluorescens* CHA0 and In5 and other cyanogenic *P. protegens* strains, to overlap each other (Michelsen and Stougaard 2012).

In *P. corrugata* CFBP 5454 cyanogenesis seems to be not regulated either by AHL-QS or by RfiA because of mutants still retained the ability to produce HCN and no *lux* box-like element was found in the *hcnABC* promoter sequence of the HCN operon. Similarly in *P. fluorescens* In5 and in *P. protegens* CHA0 no *lux*-box like element was found (Blumer and Haas 2000a, Michelsen and Stougaard 2012). Regulation of HCN production by the QS regulators LasR and RhlR was instead demonstrated in *P. aeurginosa* PAO1 (Pessi and Haas, 2000).

Volatile compounds such as ammonia and HCN are produced by several rhizobacteria and are involved in biocontrol (Brimecombe *et al.,* 2001). To investigate on the role of HCN in the two *Pseudomonas* strains biocontrol activity we performed *in vitro* experiments in which the *P. corrugata hcnA* and *hcnC* knockout mutants were cultivated together with *B. cinerea* in dual-culture experiments on Petri dishes. Although the inhibition activity of the WT strain on mycelia growth was reduced in *hcn-* mutants the most relevant effect was on conidia germination that was almost completely restored in the mutants. After two days of exposition to the WT volatiles when the plates containing bacteria and fungi were separated germination was restored thus suggesting fungistatic role of HCN. Moreover since the *hcn-* mutants did not fully restore the WT phenotype the involvement of other volatiles cannot ruled out. *P. corrugata* strain NRRL B-30409 was

demonstrated to produce ammonia but not HCN, so a strain dependent phenotype could be possible (Trivedi *et al.,* 2008).

In conclusion we showed that *P. corrugata* and *P. mediterranea* produce cyclic lipopeptides and siderophore with antimicrobial activity *in vitro*. However, the antagonistic activity pattern. The availability of the *P. corrugata* genome, associated with a systematic search of biosynthetic clusters involved in antimicrobial compounds production, and further *in vivo* tests, will allow us to develop and optimize new biocontrol strategies.

6 . Conclusions

P. corrugata (Roberts and Scarlett) emend. Sutra *et al* . (1997) and *P. mediterranea* (Catara *et al*., 2002) are closely related bacteria investigated as causal agents of tomato pith necrosis worldwide and more recently evaluated as biocontrol agents and producers of industrially-promising biomolecules for potential applications in different fields.

The interest of our work focused mainly on CLPs that are surface active molecules with antibacterial, antifungal, citotoxic and phytopathogenic properties (Bender, 1999; Raaijmaker *et al.,* 2006). Moreover lipopeptide properties may lead to applications in diverse areas of industry. Those produced by plant associated *Pseudomonas* are often thought to be useful for agro-industrial uses such as the development of microbiological pesticides or the production of biopesticides. Nonetheless CLPs are also studied for their exploitation in pharmaceutical industry, lipopeptides could been used when conventional antibiotics were no longer working against resistant bacteria or fungi; in the cosmetic industry, surfactant and anti-wrinkle characteristics of lipopeptides are applied in dermatological products; in food production, lipopeptides are used as emulsifiers in various foodstuffs and as biosurfactants, giving rise to several industrial and medical applications (Mandel *et al.,* 2013).

P. corrugata is known to produce two kinds of cyclic lipopeptides, corpeptins and cormycin A. Corpeptins were the only CLPs isolated from the culture filtrates of the type strain NCPPB 2445 (Emanuele *et al*., 1998) but later a strain dependent production of the nonapeptides cormycin was demonstrated in the strain PVCT 10.3 (Scaloni *et al*., 2004). Purified CLPs showed both phytotoxic and antimicrobial activity thus a role was suggested both in virulence and biocontrol activity.

Scarce information was available on molecular aspect of the biology of *P. corrugata* and only years later investigations on its Quorum sensing (QS) system and the transcriptional regulator RfiA helped to postulate a role on virulence mechanism mediated by CLPs during the detrimental interaction with tomato plants (Licciardello *et al*., 2007, 2009). On the contrary no information was available on *P. mediterranea*/tomato molecular interaction, or any differences with the closely related taxon *P. corrugata* were known. Both species are able to induce on tomato undistinguishable symptoms thus we thought that is likely that they share common virulence mechanisms. QS allows bacterial population to coordinate and regulate expression of some traits in a cell-density dependent manner, using signal

molecules acyl-homoserine-lactones (AHLs). The AHL-QS relies on two proteins, an AHL-synthase member of the LuxI family and the cognate receptor belonging to the LuxR family.

Starting from these premises we investigated if *P. mediterranea* also possesses a QS system. It was established that *P. mediterranea* CFBP 5447 harbours one system designated PmeI/R that is highly homologous to the PcoI/R system of *P. corrugata*. Here we demonstrated that virulence on tomatoes is affected by inactivation of QS genes in both species but with some differences, revealed particularly by the comparison of "I" mutants. Moreover, a LuxR regulator RfiA, encoded by *rfiA* that is cotranscribed with the *luxI* homologs of both species was also demonstrated to be essential for virulence. The MALDI-TOF mass spectra of bacterial cell-free culture filtrates showed that *P. corrugata* CFBP5454 produces both corpeptins and cormycin and for the first time these CLPs were also found in a *P. mediterranea* strain which spectrum also showed a putatively unidentified bioactive compound having mass weight of 1366.

Quorum sensing derivative mutant strains of both species, *P. mediterranea* CFBP5447 *pmeI*- and *pmeR*-, *P. corrugata* CFBP 5454 *pcoR*- and both *rfiA*- mutants, were impaired in CLP production as revealed by their MALDI-TOF mass spectra and also showed a drastic reduction in virulence when inoculated in tomato plants demonstrating their pivotal role in developing necrosis in tomato stem pith. Colony morphology in those mutants turned from wrinkled, or corrugated hence name of the species, and cream yellow to smooth and creamy white. More importantly another associated phenotype was the drastic reduction of the biosurfactant activity. These results also evidence that the QS is involved in CLP production and all the correlated phenotypes via the RfiA transcriptional regulator.

The absence of further genetic and molecular information and since genes regulated by QS are often located in its neighborhood we sequenced the complete *P. corrugata* CFBP 5454 cosmid insert from which the AHL QS genes were identified to further achieve information on CLP production. This allowed us to describe genes encompassing approximately 10 kb of DNA which we designated *crpC*, *crpD*, *crpE* and that we demonstrated work as an operon. Analysis of the 6.4-kb region encompassing the 3' end of *crpC* indicates that this gene codes for a non ribosomal peptide synthetase. Downstream of *crpC*, *crpDE* encoded for an ABC transporter system, consisting of a cytoplasmic membrane fusion protein (MFP) *crpD* and ABC transporter (ABCt) *crpE* , which shows similarity to macrolide efflux systems of other *Pseudomonas* spp. (MacA/B homologs).

Mutational analysis of *crpC* and *crpDE* demonstrated that these genes are involved in the production and secretion of corpeptins since the derivative mutant strain cell-free culture filtrates showed only the peaks of cormycin in their MALDI-TOF spectra. Moreover, pathogenicity tests revealed that, as syringopeptins for *P. syringae*, this long chain CLPs are important for *P. corrugata* virulence. Using real-time PCR we also ascertained that the PcoR and RfiA regulators are involved in corpeptins production/secretion. Moreover, the time-course expression analysis in the parent strain highlighted that corpeptin transcription occurs at the achievement of the *quorum* thus the gene expression follows the AHL signal molecules production trend, reaching high amounts of transcripts as consequence of the positive-feedback regulatory loop.

The role of QS in CLP production was demonstrated in a number of saprophytic *Pseudomonas* but it was not defined clearly in phytopathogenic *Pseudomonas* spp. (D'aes *et al.,* 2010). In *P. syringae* the production of syringopeptins and syringomycin is not controlled by QS but by the two component GacA/GacS system through the non-QS LuxR-type transcriptional activators SalA and SyrF (Lu *et al.*, 2005). Genomic inactivation of non-QS LuxR-type regulatory genes in *P. fluorescens* SBW25 resulted in viscosin deficiency and reduced transcript levels of viscosin biosynthesis genes (de Bruijin and Raaijmakers, 2009).

The last part of this work was devoted to investigate if the CLPs have also a role in the biological control activity of *P. corrugata* and *P. mediterranea.* Since the antagonistic activity of *Pseudomonas* is linked to direct mechanisms of competition, antibiosis or parasitism and indirect effects such as induction of resistance that are mostly affected by the production of secondary metabolites (Haas and Défago, 2005) the area of investigation was extended.

We ascertained that *P. corrugata* CFBP 5454 and *P. mediterranea* CFBP5447 produce *in vitro* diffusible compounds with antimicrobial properties and substances whose activity is ascribable to competition for iron which inhibited the growth of either plant pathogenic fungi and bacteria, as well as volatile compounds able to inhibit the fungal mycelial growth and sporulation. Only the *P. corrugata* strain was able to antagonize *X. campestris* pv. *campestris.* The cell-free culture filtrates produced from cultures grown in CLP induction conditions showed antimicrobial activity towards the CLP indicator microorganisms the Gram-positive bacterium *Bacillus megaterium* and the yeast *Rhodotorula pilimanae.*

To further investigate on the molecular mechanism of the antimicrobial activity we also tested the QS and the mutants in the gene encoding the RfiA transcriptional regulator of both species. By analyzing the antagonistic activity against phytopathogenic fungi and

bacteria no differences were observed between the QS mutants and their respective parent strains whereas antifungal activity was completely abolished in the *rfiA*- mutants. Since both the *pcoR*- (and *pmeR*-) and *rfiA*- mutants did not produce CLPs the production of one or more additional antimicrobial compound is suggested. Thus, mutational analysis revealed that RfiA has an important role in interaction with the plant and microorganisms *via* the production of CLPs and other unknown metabolites.

Finally, we set up a trial to investigate the role of CLPs in biocontrol in an *in vivo* experiment. The CLP role is controversial and dependent on pathosystems analyzed, since has been shown that biocontrol agents may produce CLPs but in some cases these molecules are not required for disease suppression (Tran *et al* ., 2007; Mazzola *et al* ., 2007; Berry *et al* ., 2010). This seems the case of *P. corrugata* since its *pcoR*- and *rfiA*- mutants are still able to reduce the disease incidence in the *in vivo* investigated pathosystems. This finding could have a positive applicative significance because if CLP are pivotal for virulence, which it is not the same for biocontrol, thus strains that produce low amounts of CLP or even do not produce them anymore could be applied as biological control agent without potential risk for susceptible plant species. However, it must be taken into account that CLP activity could be specific for certain pathogens (e.g. they lyses *Pythium* zoospores) or crucial for bacterial colonization and establishment due to their role in motility and biofilm formation.

Only very recently the availability of the draft genome sequence of *P. corrugata* CFBP 5454 helped in mining for secondary metabolite clusters[1] (Catara *et al.,* 2013). Mining by BLAST using as baits genes encoding a spectrum of traits involved in biological control and identified in the genomes of several sequenced *P. fluorescens* strains (Loper *et al*, 2012) revealed that it does not have genes involved in the synthesis of antimicrobial compounds like phenazines, pyoluteorin, pyrrolnitrin, or 2,4-diacetylphloroglucinol (data not shown) but has a biosynthetic cluster for the enzyme HCN synthase involved in this volatile compound biosynthesis.

[1]GenBank Accession no: ATKI00000000. De novo genome sequencing of *Pseudomonas corrugata* phytopathogenic bacterium and biological resource". Licciardello G., Jackson W.R, Bella P., Strano C. P., Catara A. F., Arnold D. L., Venturi V., Silby M.W., Catara V. (2013, submitted).

The HCN synthesis gene cluster was identified in strain CFBP 5454, and DNA sequence analysis showed similarity to known *hcnABC* gene clusters. *P. corrugata hcnA* and *hcnC* knockout mutants failed to produce HCN and showed a reduced activity against *Botrytis cinerea* above all against conidia germination. Cyanogenesis seems to be not regulated either by AHL-QS or by RfiA because of mutants still retained the ability to produce HCN and no *lux* box-like element was found in the *hcnABC* promoter sequence.

A top-down approach is now aiming in finding new natural products in these two intriguing bacteria by mining their genome by bioinformatics. Currently, a large number of potential secondary metabolite biosynthetic gene clusters were predicted using AntiSMASH v2.0 (Blin *et al.,* 2013), which may contribute to bacterial fitness, competitiveness and phytopathogenicity. In *P. corrugata* a total of 217 kb (3.5% of the genome) were involved in putative non ribosomal peptide sythethase gene clusters which belong to at least three biosynthetic clusters one of which may be associated with the production of the siderophore corrugatine (Risse *et al.*, 1998). The other two showed high homology in both domain structures and sequences to clusters associated with the production of syringomycin and syringopeptins in *P. syringae* (Wang *et al.*, 2006). We therefore predict these to be involved in the synthesis of the two classes of CLPs described in *P. corrugata*, cormycin and corpeptins. In addition a number of other secondary metabolite biosynthetic clusters including hitherto-unknown putative toxic/antimicrobial metabolites were revealed that could be responsible for the bioactivity evidenced in our study and that turns to be of potential interest for biotechnological applications.

Ringraziamenti

L'esperienza del dottorato richiede sicuramente passione, dedizione, impegno, ed ottenere risultati spesso non è sempre così immediato per cui è richiesta anche una buona dose di testardaggine e caparbietà. Nella mia esperienza ho imparato che oltre tutto questo è fondamentale "vivere col cuore" il dottorato, perché solo così lo si può rendere un'esperienza unica. L'amicizia e le collaborazioni sincere sono alla base della buona riuscita di qualsiasi lavoro e quindi mi è doveroso ringraziare tutti coloro che hanno diversamente contribuito alla buona riuscita della mia esperienza e alla mia crescita umana.

Non è da tutti accogliere una perfetta sconosciuta e accordargli subito fiducia. Questa è la Prof.ssa Vittoria Catara alla quale va il ringraziamento più grande e per la quale nutro la stima più incondizionata. Esempio di professionalità e di profonda umanità, a cui devo l'insegnamento professionale e di vita più importante.

Un grazie va anche alla Dott.ssa Patrizia Bella con la quale ho condiviso innumerevoli caffè, ed intense ore e giornate in laboratorio e alla Dott.ssa Grazia Licciardello per avermi pazientemente istruita al mio arrivo in questo gruppo di ricerca.

Ringrazio il Dott. Vittorio Venturi e la Dott.ssa Iris Bertani (ICGEB di Trieste), il Prof. Vincenzo Fogliano ed il Dott. Alberto Fiore (Università di Napoli) e la Prof.ssa Angela Roberta Lo Piero (Università di Catania), per avermi seguita durante i soggiorni presso i loro laboratori e per la loro continua collaborazione nella messa a punto degli esperimenti e nell'interpretazione dei risultati.

Un doveroso ringraziamento al Professor Antonino Catara, per i consigli e suggerimenti e aver supportato scientificamente questa ricerca attraverso la collaborazione a svariati progetti.

Un ringraziamento speciale va a tutti i miei colleghi ed amici da cui ho imparato l'importanza del condividere le esperienze, di un abbraccio e del sorridere, ed il valore profondo dell'amicizia. Anche questo ha contribuito alla mia crescita umana.

Infine, ma non per questo ultimi, il GRAZIE più profondo ai miei genitori e a mia sorella, i sostenitori più grandi di questo mio progetto ed esperienza di vita, guida e sostegno senza i quali sicuramente non sarei la persona che sono. A loro devo tutto ed un grazie non sarà mai abbastanza.

REFERENCES

Achouak, W., Thie'ry, M., Roubaud, P. and Heulin, T. (2000). Impact of crop management on intraspecific diversity of *Pseudomonas corrugata* in bulk soil. *FEMS Microbiol. Ecol.* 31, 11–19.

Alexeyev, M. F. (1999) The pKNOCK series of broad-host-range mobilizable suicide vectors for gene knockout and targeted DNA insertion into the chromosome of gram-negative bacteria. *BioTechniques* **26**, 824-

Alippi, A. M., Lòpez, A.C. (2010). First report of *Pseudomonas mediterranea* causing tomato pith necrosis in Argentina. *Plant Pathol.* **59**, 1163. 828.

Allison C, Lai HC, Gygi D & Hughes C (1993) Cell differentiation of Proteus mirabilis is initiated by glutamine, a specific chemoattractant for swarming cells. *Mol Microbiol* **8**: 53–60.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990) Basic local alignment search tool. *J. Mol. Biol.* **215**, 403-410.

Ait Tayeb, L., Ageron, E., Grimont, F. and Grimont, P.A.D. (2005). Molecular phylogeny of the genus *Pseudomonas* based on *rpoB* sequences and application for the identification of isolates. *Res. Microbiol.* **156**, 763–773.

Andersen, J. B., Koch, B., Nielsen, T. H., Sørensen, D., Hansen, M., Nybroe, O., Christophersen, C., Sørensen, J., Molin, S., and Givskov, M. (2003). Surface motility in *Pseudomonas* sp. DSS73 is required for efficient biological containment of the root-pathogenic microfungi *Rhizoctonia solani* and *Pythium ultimum*. *Microbiology* **149**:37-46.

Anzai, Y., Kim, H., Park, J.Y., Wakabayashi, H. and Oyaizu, H. (2000). Phylogenetic affiliation of the pseudomonads based on 16S rRNA sequence. *Int. J. Syst. Evol. Microbiol.* **50**, 1563–1589.

Arima, K., Imanaka, H., Kousaka, M., Fukuda, A. & Tamura, G. (1964). Pyrrolnitrin, a new antibiotic substance, produced by *Pseudomonas***.** *Agr Biol Chem* **28,** 575-576.

Arrebola, E., Cazorla, F.M., Durán, V.E., Rivera, E., Olea, F., Codina, J.C., Pérez-García, A., de Vicente, A. (2003). Mangotoxin: a novel antimetabolite toxin produced by *Pseudomonas syringae* inhibiting ornithine/arginine biosynthesis. *Physiol. Mol. Plant Pathol.* **63**:117–127.

Arrebola, E., Cazorla, F.M., Romero, D., Pérez-García, A., de Vicente, A. (2007). A nonribosomal peptide synthetase gene (*mgoA*) of *Pseudomonas syringae* pv. *syringae* is involved in mangotoxin biosynthesis and is required for full virulence. *Mol. Plant-Microbe Interact.* **20**:500 –509.

Arrebola, E., Cazorla, F.M., Codina, J.C., Gutiérrez-Barranquero, J.A., Pérez- García, A., de Vicente, A. (2009). Contribution of mangotoxin to the virulence and epiphytic fitness of *Pseudomonas syringae* pv. *syringae. Int. Microbiol.* **12**:87–95.

Arrebola, E., Cazorla, F.M., Pérez-García, A., de Vicente, A. (2011). Genes involved in the production of antimetabolite toxins by *Pseudomonas syringae* pathovars. *Genes* **2**:640–660.

Arrebola, E., Carrión, V.J., Cazorla, F.M., Pérez-García, A., Murillo, J., de Vicente, A. (2012). Characterisation of the *mgo* operon in *Pseudomonas* syringae pv. *syringae* UMAF0158 that is required for mangotoxin production**.** *BMC Microbiol.* 12:10. doi:10.1186/1471-2180-12-10.

Ashby, R.D., Solaiman, D.K.Y. and Foglia, T.A. (2003). Bacterial poly (hydroxyalkanoate) polymer production from the biodiesel co-product stream. *J. Polymer. Environ.* **12**, 105–112.

Ashby, R.D., Solaiman, D.K. and Foglia, T.A. (2005). Synthesis of short-/medium-chain-length poly (hydroxyalkanoate) blends by mixed culture fermentation of glycerol. *Biomacromolecules.* **6**, 2106–2112.

Bais HP, Fall R , Vivanco JM (2004) Biocontrol of *Bacillus subtilis* against infection of Arabidopsis roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. *Plant Physiol* **134**: 307–319.

Balibar, C.J., Vaillancourt, F.H., Walsh, C.T. (2005) Generation of D amino acid residues in assembly of arthrofactin by dual condensation/epimerization domains. *Chem Biol* **12**: 1189–1200.

Ballio, A., Barra, D., Bossa, F., Collina, A., Grgurina, I., Marino, G., Moneti, G., Paci, M., Pucci, P., Segre, A., and Simmaco, M. (1991). Syringopeptins, new phytotoxic lipodepsipeptides of *Pseudomonas syringae* pv. *syringae*. *FEBS Lett.* **291,** 109-112.

Ballio, A., Bossa, F., Camoni, L., Di Giorgio, D., Flamand, M. C., Maraite, H., Nitti, G., Pucci, P., Scaloni A. (1996) Structure of fuscopeptins, phytotoxic metabolites of *Pseudomonas fuscovaginae*. *FEBS Lett.* **381**, 213-216.

Baltrus, D.A., Nishimura, M.T., Romanchuk, A., Law,T., Chang, J.H., Muhktar, S., Jones, C.D., Dangl, J.L. Dynamic evolution of pathogenicity revealed by sequencing and comparative genomics of 19 *Pseudomonas syringae* isolates. Unpublished.

Barber, C.E., Tang, J.L., Feng, J.X., Pan, M.Q., Wilson, T.J., Slater, H., Dow, J.M., Williams, P., Daniels, M.J. (1997). A novel regulatory system required for pathogenicity of *Xanthomonas campestris* is mediated by a small diffusible signal molecule. *Mol Microbiol* **24** (3):555-66.

Bare, S., Coiro, V. M., Scaloni, A., Di Nola, A., Paci, M., Segre, A. L., Ballio, A. (1999). Conformations in solution of the fuscopeptins—phytotoxic metabolites of *Pseudomonas fuscovaginae*. *Eur. J. Biochem*. **266**:484-492.

Barnard, A. M. L., and Salmond, G. P. C. (2007). Quorum sensing in *Erwinia* species. *Anal. Bioanal. Chem.* **387**:415-423.

Barnett, J.S., Singleton, I., Ryder, M. (1998). Spatial variation in populations of *Pseudomonas corrugata* 2140 and pseudomonads on Take-all diseased and healthy root systems of wheat. *Soil Biol. Biochem*. **31**, 633–636.

Barnard, A. M. L., Salmond, G. P. C. (2007). Quorum sensing in *Erwinia* species. Anal. *Bioanal. Chem.* **387**, 415-423.

Basim, H., Basim, E., Yilmaz, S. and Ilkucan, M. (2005) First report of Pith Necrosis of tomato caused by *Pseudomonas mediterranea* in Turkey. *Plant Pathol*. **54**, 240.

Bassler, B.L. (2002). Cell-to-cell communication in bacteria. *Cell* **109**: 421–424.

Bell, C.R., Dickie, G.A., Chan, J.W.Y.F. (1995). Variable response of bacteria isolated from grapevine xylem to control grape crown gall disease in planta. *Am. J. Enol. Vitic.* **46**, 499–508.

Bella, P., Licciardello, G., Lombardo, A., Pitman, A., Arnold, D. L., Solaiman, D. K. Y., Catara, V. (2007). Cloning and sequencing of *Pseudomonas mediterranea* PHA locus. 4th European Symposium on Biopolymers, Turkey, Book of Abstract, 131.

Bender, C.L., Alarcon-Chaidez, F., Gross, D.C. (1999). *Pseudomonas syringae* phytotoxins: mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. *Microbiol. Mol. Biol. Rev.* **63**,266-292.

Bensaci M. F., Gurnev P.A.,, Bezrukov S.M., and Takemoto J.Y. (2011). Fungicidal activities and mechanisms of action of *Pseudomonas syringae* pv. *syringae* lipodepsipeptide syringopeptins 22A and 25A. *Frontiers in microbiology*, 2 (16), 1-7.

Berry C., Fernando W.G. Dilantha , Loewen P. C. ,. de Kievit T. R (2010) Lipopeptides are essential for *Pseudomonas* sp. DF41 biocontrol of *Sclerotinia sclerotiorum. Biological Control* **55:** 211–218.

Bertani, I., Rampioni, G., Leoni, L., Venturi, V. (2007). The *Pseudomonas putida Lon* protease is involved in N-acyl homoserine lactone quorum sensing regulation. *BMC Microbiol*. **7**, 71.

Berti, A.D.; Greve, N.J.; Christensen, Q.H.; Thomas, M.G. (2007). Identification of a biosynthetic gene cluster and the six associated lipopeptides involved in swarming motility of *Pseudomonas syringae* pv. *tomato* DC3000. *J. Bacteriol.* **189**, 63126323.

Better, M., Lewis, B., Corbin, D., Ditta, G., Helinski, D.R. (1983). Structural relationships among *Rhizobium meliloti* symbiotic promoters. *Cell* **35**, 479–485.

Bidwai, A.P. ,Zhang, L., Bachmann,R. C., Takemoto, J.Y. (1987). Mechanism of action of *Pseudomonas syringae* phytotoxin, syringomycin: stimulation of red beet plasma membrane ATPase activity. *PlantPhysiol.* **83**, 39–43.

Biemans-Oldehinkel, E., Doeven, M.K. and Poolmain, B. (2006) ABC transporter architecture and regulatory roles of accessory domains. *FEBS Lett* **580**, 1023–1035.

Blin K., Marnix H. Medema, Daniyal Kazempour, Michael A. Fischbach, Rainer Breitling, Eriko Takano, and Tilmann Weber (2013) antiSMASH 2.0—a versatile platform for genome mining of secondary metabolite producers. *Nucl. Acids Res.* doi:10.1093/nar/gkt449.

Blumer, C., and Haas, D. (2000a). Iron regulation of the *hcnABC* genes encoding hydrogen cyanide synthase depends on the anaerobic regulator ANR rather than on the global activator GacA in *Pseudomonas fluorescens* CHA0. *Microbiology*, **146**(10): 2417–2424. PMID:11021918.

Birnboim, H. C. (1983) A rapid alkaline extraction method for the isolation of plasmid DNA.*Methods Enzymol.* **100**, 243–255.

von Bodman, S.B. and Farrand, S.K. (1995). Capsular polysaccharide biosynthesis and pathogenicity in *Erwinia stewartii* require induction by an *N*-acylhomoserine lactone autoinducer. *J. Bacteriol.* **177**:5000-5008.

von Bodman, S.B., Bauer, W.D., Coplin, D.L. (2003). Quorum sensing in plant- pathogenic bacteria. *Annu. Rev. Phytopathol.* **41**, 455-482.

Brimecombe MJ, De Liej FA, Lynch JM.(2001) The effect of root exudates on rhizosphere microbial populations. In: Pinton R, Varanini Z, Nannipieri P, editors. *The Rhizosphere*. New York: Marcel Dekker. p. 95–140.

Brooks, D.M., Bender, C.L., Kunkel, B.N. (2005) The *Pseudomonas syringae* phytotoxin coronatine is required to overcome salicylic acid mediated defenses in *Arabidopsis thaliana*. *Mol Plant Pathol* **6**: 629-952 639.

Budzikiewicz, H. (2004). Siderophores of the Pseudomonadaceae *sensu stricto* (fluorescent and non-fluorescent *Pseudomonas* spp.). Prog. Chem. Org. Nat. Prod. **87**, 81D237.

Buell, C.R., Joardar, V., Lindeberg, M., Selengut, J., Paulsen, I.T., Gwinn, M.L., Dodson, R.J., Deboy, R.T., Durkin, A.S., Kolonay, J.F., Madupu, R., Daugherty, S., Brinkac, L., Beanan, M.J., Haft, D.H., Nelson, W.C., Davidsen, T., Zafar, N., Zhou, L., Liu, J., Yuan, Q., Khouri, H., Fedorova, N., Tran, B., Russell, D., Berry, K., Utterback, T., Van Aken, S.E., Feldblyum, T.V., D'Ascenzo, M., Deng, W.L., Ramos, A.R., Alfano ,J.R., Cartinhour, S., Chatterjee, A.K., Delaney, T.P., Lazarowitz, S.G., Martin, G.B., Schneider, D.J., Tang, X., Bender, C.L., White, O., Fraser, C.M. and Collmer, A. (2003). The complete genome sequence of the Arabidopsis and tomato pathogen *Pseudomonas syringae* pv. *tomato* DC3000. *Proc. Natl. Acad. Sci.* U.S.A. **100** (18), 10181-10186.

Bull, C. T., M. L. Wadsworth, K. N. Sorensen, J. Y. Takemoto, R. K. Austin, and J. L. Smilanick. 1998. Syringomycin E produced by biological control agents controls green mold on citrus*. Biol. Control* **12:**89–95.

Bultreys A and Gheysen I. (1999). Biological and molecular detection of toxic lipodepsipeptide- producing Pseudomonas syringae strains and PCR identification in plants. Appl Environ Microbiol 65: 1904–1909.

Bultreys A, Gheysen I, Maraite H and de Hoffmann E.(2001). Characterization of fluorescent and nonfluorescent peptide siderophores produced by *Pseudomonas syringae* strains and their potential use in strain identification. *Appl Environ Microbiol* **67**: 1718–1727.

Carrión, V. J., Gutiérrez-Barranquero, J. A., Arrebola,E., Bardaji, L., Codina, J. C., de Vicente, A., Cazorla, F. M., Murilloc, J. (2013). The Mangotoxin Biosynthetic Operon (*mbo*) Is Specifically Distributed within *Pseudomonas syringae* Genomospecies 1 and Was Acquired Only Once during Evolution. *Appl. Environ. Microbiol.* **79**(3): 756-767.

Catara, V., Gardan, L., Lopez, M.M. (1997) Phenotypic heterogeneity of *Pseudomonas corrugata* strains from southern Italy. *J. Appl. Microbiol.* **83**,576-586.

Catara, V., Arnold, D., Cirvilleri, G., Vivian, A. (2000) Specific oligonucleotide primers for the rapid detection of the causal agent of tomato pith necrosis, *Pseudomonas corrugata*: evidence for two genomic groups. *Eur. J. Plant Pathol.* **106**, 753–762.

Catara, V., Sutra, L., Morineeau, A., Achouak, W., Christan, R., Gardan, L. (2002). Phenotypic and genomic evidence for the revision of *Pseudomonas corrugata* and proposal of *Pseudomonas mediterranea* species sp. nov. *Int. J. Syst. Evol. Microbiol.* **52**, 1749-1758.

Catara, V. (2007). *Pseudomonas corrugata*: plant pathogen and/or biological resource? *Mol. Plant Pathol.* **8**, 233-244.

Catara V., Licciardello G., Bella P., Strano C.P., Catara A.F., Arnold D.L., Venturi V., Silby M.W., Jackson R.W. (2013). Genome sequencing and annotation of the plant associated bacterium *Pseudomonas corrugata*. XIX Congress of S.I.Pa.V. 23-25/09, Padua, Italy. Book of Abstract, p.19

Cazorla, F.M., Arrebola, E., del Moral, E., Rivera, E., Olea, F., Pérez-García, A.,de Vicente, A. (2003) An antimetabolite toxin (mangotoxin) is produced by *Pseudomonas syringae* pv. *syringae* isolated from mango. In: Iacobellis NS, et al (eds) *Pseudomonas syringae* and related pathogens: biology and genetics. *Kluwer Academic Pub.,* Dordrecht, pp 175-183.

Cirvilleri, G., Bella, P., Catara, V. (2000). Luciferase genes as a marker for *Pseudomonas corrugata. J. Plant Pathol.* **82**, 237–241.

Cirvilleri, G., Bella, P., Catara, V. (2001). Biocontrol activity in plant pathogenic *Pseudomonas* species. In: *Proceedings 5th Congress of the European Foundation for Plant Pathology*, pp. 534–538.

Cirvilleri, G., Bonaccorsi, A., Scuderi, G., and Scortichini, M. 2005. Potential biological control activity and genetic diversity of *Pseudomonas syringae* pv. *syringae* strains. *J. Phytopathol.* 153:654-666.

Cladera, A.M., Sepulveda-Torres, L.C., Valens-Vadell, M., Meyer, J.M., Lalucat, J., Garcia-Valdes, E. (2006). A detailed phenotypic and genotypic description of *Pseudomonas* strain OX1. *Syst. Appl. Microbiol.* **29**, 422-430.

Che, F.-S., K. Kasamo, N. Fukuchi, A. Isogai, and A. Suzuki. 1992. Bacterial phytotoxins, syringomycin, syringostatin and syringotoxin, exert their effect on the plasma membrane H1-ATPase partly by a detergent action and partly by inhibition of the enzyme. *Physiol. Plant.* **86:**518–524.

Chen, C., Bélanger, R.R., Benhamou, N., Paulitz, T.C. (1999). Role of salicylic acid in systemic resistance induced by *Pseudomonas* spp. against *Pythium aphanidermatum* in cucumber roots. *Eur. J. Plant Pathol.* **105**, 477–486.

Cho, H. and Kang, H. (2012). The PseEF efflux system is a virulence factor of *Pseudomonas syringae* pv. *syringae J. Microbiol.* **50**(1), 79–90.

Choi, H.Y., Ryder, M.H., Gillings, M.R., Stokes, H.W., Ophel-Keller, K.M., Veal, D.A. (2003). Survival of a *lacZY*-marked strain of *Pseudomonas corrugata* following a field release. *FEMS Microbiol. Ecol.* **43**, 367–374.

Chun, W. and Leary, J.V. (1989) Novel toxin produced by *Pseudomonas corrugata*, the causal agent of tomato pith necrosis. In: *Phytotoxins and Plant Pathogenesis* (Graniti, A., Durbin, R.D. and Ballio, A., eds), pp. 93– 116. Berlin: Springer-Verlag.

Clarke, C.R., Cai, R., Studholme, D.J., Guttman, D.S. and Vinatzer, B.A. (2010.) *Pseudomonas syringae* strains naturally lacking the classical *P. syringae hrp/hrc* locus are common leaf colonizers equipped with an atypical type III secretion system. *Mol. Plant Microbe Interact.* **23** (2), 198-210.

Cliften, P., Y. Wang, D. Mochizuki, T. Miyakawa, R. Wangspa, J. Hughes, and J. Y. Takemoto. 1996. *SYR2*, a gene necessary for syringomycin growth inhibition of *Saccharomyces cerevisiae*. *Microbiology* **142:**477–484.

Coraiola, M., Lo Cantore, P., Lazzaroni, S., Evidente A., Iacobellis, N.S. and Dalla Serra, M. (2006). WLIP and tolaasin I, lipodepsipeptides from *Pseudomonas reactans* and *Pseudomonas tolaasii*, permeabilise model membranes. *Biochimica et Biophysica Acta* **1758**, 1713–1722.

Coraiola, M., Paletti, R., Fiore, A., Fogliano, V., and Dalla Serra V. (2008). Fuscopeptin, antimicrobial lipodepsipeptides from *Pseudomonas fuscovaginae*, are channel forming peptides active on biological and model membranes. *J. Pept. Sci.* **14**, 496–502.

Cornelis P., and Matthijs S. (2002). Diversity of siderophore-mediated iron uptake systems in fluorescent pseudomonads: not only pyoverdines. *Environmental Microbiology* **4**(12), 787–798.

Cornelis P., and Matthijs S. (2007). *Pseudomonas* Siderophores and their Biological Significance. Soil Biology, Volume 12 Microbial Siderophores A. Varma, S.B. Chincholkar (Eds.).

Corsaro, M.M., Evidente, A., Lanzetta, R., Lavermicocca, P., & Molinaro, A. (2001). Structural determination of the phytotoxic mannan exopolysaccharide from *Pseudomonas syringae* pv. *ciccaronei*. *Carbohydrate Research*, 330, 271-277.

Conte, E., Catara, V., Greco, S., Russo, M., Alicata, R., Strano, L., Lombardo, A., Di Silvestro, S. and Catara, A. (2006). Regulation of polyhydroxyalkanoate synthases (*phaC1* and *phaC2*) gene expression in *Pseudomonas corrugata. Appl Microbiol Biotechnol* **72** (5), 1054-62

Cox, C. D., Rinehart, K. L., Moore Jr M. L., Cook, Jr J. (1981). Pyochelin: novel structure of an iron-chelating growth promoter for *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **78**: 4256–4260.

Cui, X., Harling, R., Mutch, P., and Darling, D. (2005). Identification of *N*-3- hydroxyoctanoyl-homoserine lactone production in *Pseudomonas fluorescens*5064, pathogenic to broccoli, and controlling biosurfactant production by quorum sensing. Eur. *J. Plant Pathol.* **111**, 297–308.

Dalla Serra, M., Fagiuoli, G., Nordera, P., Bernhart, I., Della Volpe, C., Di Giorgio, D., Ballio, A. and Menestrina, G. (1999). The interaction of lipodepsipeptide toxins from *Pseudomonas syringae* pv. *syringae* with biological and model membranes: a comparison of syringotoxin, syringomycin and syringopeptins. *Mol. Plant Microbe Interact.* **12**, 391–400.

Dalla Serra, M., Menestrina, G., Carpaneto, A., Gambale, F., Fogliano, V. and Ballio, A. (2003). Molecular mechanism of action of syringopeptins, antifungal peptides from *Pseudomonas syringae* pv. *syringae*. In Pore-Forming Peptides and Protein Toxins, vol. 5 (Menestrina, G., Dalla Serra, M. and Lazarovici, P., eds.; Cellular and Molecular Mechanisms of Toxin Action, Lazarovici, P., series ed.), pp. 272–295, Taylor & Francis Group, London

D'aes, J., De Maeyer, K., Pauwelyn, E., Höfte, M. (2010). Biosurfactants in plant–*Pseudomonas* interactions and their importance to biocontrol. *Environ. Microbiol. Rep*. **2**, 359–372.

D'aes, J., Ongena, M. and Höfte, M. (2011). Cyclic lipopeptides of *Pseudomonas* sp.CMR12a play contrasting roles in swarming motility and biofilm formation. 63rd International Symposium on Crop Protection.

D'aes, J. (2012). Biological activity and regulation of cyclic lipopeptides and phenazines produced by biocontrol strain *Pseudomonas* CMR12a. PhD thesis. Ghent University. Faculty of Bioscience Engineering, Ghent, Belgium.

Daniels, R., Vanderleyden, J., Michiels, J. (2004). Quorum sensing and swarming migration in bacteria. *FEMS Microbiol. Rev.* **28**, 261–289.

De Bruijn, I., de Kock, M.J.D., Yang, M., de Waard, P., van Beek, .TA. and Raaijmakers,J.M. (2007). Genome-based discovery, structure prediction and functional analysis of cyclic lipopeptide antibiotics in Pseudomonas species. *Mol Microbiol* **63**, 417–428.

De Bruijn, I., de Kock, M. J. D., de Waard, P., van Beek, T. A. and Raaijmakers J. M. (2008). Massetolide A biosynthesis in *Pseudomonas fluorescens. .J. Bacteriol*. **190**, 2777–2789.

De Bruijn, I. and Raaijmakers, J.M. (2009a). Diversity and functional analysis of LuxR-type transcriptional regulators of cyclic lipopeptide biosynthesis in *Pseudomonas fluorescens*. *Appl Environ Microb* **75**, 4753–4761.

De Bruijn, I. and Raaijmakers J. M. (2009b). Regulation of cyclic lipopeptide biosynthesis in *Pseudomonas fluorescens* by the ClpP protease. *J. Bacteriol*. **191:** 1910–1923.

De la Torre-Zavala S., S. Aguilera , E. Ibarra-Laclette , J. L. Hernandez-Flores, A. Herna´ndez-Morales , J. Murillo , A. Alvarez-Morales. (2011). Gene expression of Pht cluster genes and a putative non-ribosomal peptide synthetase required for phaseolotoxin production is regulated by GacS/GacA in *Pseudomonas syringae* pv. *phaseolicola. Research in Microbiology* **162,** 488-498.

De Souza, J. T., De Boer, M., De Waard, P., Van Beek, T. A., Raaijmakers, J. M. (2003). Biochemical, genetic, and zoosporicidal properties of cyclic lipopeptide surfactants produced by *Pseudomonas fluorescens*. *Appl. Environ. Microbiol*. **69**:7161-7172.

De Vos, P., Goor, M., Gillis, M. and De Ley, J. (1985). Ribosomal ribonucleic acid cistron similarities of phytopathogenic *Pseudomonas* species. Int. J. Syst. Bacteriol. **35**, 169–184.

Dietrich, L.E., Teal, T.K., Price-Whelan, A., Newman, D.K. (2008). Redox-active antibiotics control gene expression and community behavior in divergent bacteria. *Science* **321** (5893): 1203-1206.

Donadio, S., Monciardini, P., Sosio, M., (2007). Polyketide synthases and nonribosomal peptide synthetases: the emerging view from bacterial genomics. *Nat. Prod. Rep.*,**24,** 1073–1109.

Duan J, Jiang W, Cheng Z, Heikkila JJ, Glick BR (2013) The Complete Genome Sequence of the Plant Growth-Promoting Bacterium *Pseudomonas* sp. UW4. PLoS ONE 8(3): e58640. doi:10.1371/journal.pone.0058640

Dubern, J.F., Lagendijk, E.L., Lugtenberg, B.J.J. and Bloemberg, G.V. (2005). The heat shock genes *dnaK*, *dnaJ*, and *grpE* are involved in regulation of putisolvin biosynthesis in *Pseudomonas putida* PCL1445. *J Bacteriol* **187**, 5967–5976.

Dubern, J. F., Lugtenberg, B. J. J. and Bloemberg, G. V. (2006). The *ppuI-rsaL-ppuR* quorum-sensing system regulates biofilm formation of *Pseudomonas putida* PCL1445 by controlling biosynthesis of the cyclic lipopeptides putisolvins I and II. *J. Bacteriol*. **188**, 2898–2906.

Dubern, J.F., Coppoolse, E.R., Stiekema, W.J. and Bloemberg, G.V. (2008). Genetic and functional characterization of the gene cluster directing the biosynthesis of putisolvin I and II in *Pseudomonas putida* strain PCL1445. *Microbiology* **154**, 2070–2083.

Dufour, S., Deleu, M., Nott, K., Wathelet, B., Thonart, P., and Paquot, M. 2005. Hemolytic activity of new linear surfactin analogs in relation to their physico-chemical properties. *Biochim. Biophys. Acta*. 1726:87-95

Dulla, G., and Lindow, S. E. (2008). Quorum size of *Pseudomonas syringae* is small and dictated by water availability on the leaf surface. *Proc.Natl. Acad. Sci.* U.S.A. **105**: 3082-3087.

Dumenyo, C.K., Mukherjee, A., Chun, W., Chatterjee, A.K. (1998). Genetic and physiological evidence for the production of N-acyl homoserine lactones by *Pseudomonas syringae* pv. *syringae* and other fluorescent plant pathogenic *Pseudomonas* species. *Eur J Plant Pathol* **104**: 569–582.

Durbin, R. D., Uchytil, T. F., Steele, J. A., Ribeiro, L. D. (1978). Tabotoxinine β-lactam from *Pseudomonas tabaci*. *Phytochemistry*, **17**, 147–148.

Elasri, M., Delorme, S., Lemanceau, P., Stewart, G., Laue, B., Glickmann, E., Oger, P.M., Dessaux, Y. (2001). Acyl-homoserine lactone production is more common among plant-associated *Pseudomonas* spp. than among soilborne *Pseudomonas* spp. *Appl Environ Microbiol* **67**: 1198–1209.

Emanuele, M. C., Scaloni, A., Lavermicocca, P., Jacobellis, N. S., Camoni,L., Di Giorgio, D., Pucci, P., Paci, M., Segre, A., and Ballio, A. (1998). Corpeptins, new bioactive lipodepsipeptides from cultures of *Pseudomonas corrugata*. *FEBS Lett.* **433**, 317.

Feigin, A. M., J. Y. Takemoto, R. Wangspa, J. H. Teeter, and J. G. Brand. (1996). Properties of voltage-gated ion channels formed by syringomycin E in planar lipid bilayers. J. Membr. Biol. **149:**41–47.

Feil, H., Feil, W.S., Chain, P., Larimer, F., Di Bartolo, G., Copeland, A., Lykidis, A., Trong, S., Nolan, M., Goltsman, E., Thiel, J., Malfatti, S., Loper, J.E., Lapidus, A., Detter, J.C., Land, M., Richardson, P.M., Kyrpides, N.C., Ivanova, N. and Lindow,S.E. (2005). Comparison of the complete genome sequences of *Pseudomonas syringae* pv. *syringae* B728a and pv. *tomato* DC3000. *Proc. Natl. Acad. Sci.* U.S.A. **102** (31), 11064-11069.

Ferguson, A. R., and J. S. Johnston. (1980). Phaseolotoxin: chlorosis, ornithine accumulation and inhibition of ornithine carbamoyltransferase in different plants. *Physiol. Plant Pathol.* **16:**269–275.

Fernando, W.G.D., Ramarathnam, R., Krishnamoorthy, A.S., Savchuk, S.C. (2005). Identification and use of potential bacterial organic antifungal volatiles in biocontrol. *Soil Biol. Biochem.* **37**, 955–964.

Fett, W.F., & Dunn, M.F. (1989). Exopolysaccharides produced by phytopathogenic *Pseudomonas syringae* pathovars in infected leaves of susceptible hosts. *Plant Physiology*, **89,** 5-9.

Figurski, D. H. and Helinski, D. R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc. Natl. Acad. Sci.* USA **76**, 1648–1652.

Finney, A.H., Blick, R.J., Murakami, K. Ishihama, A., Stevens, A. M. (2002). Role of the C-terminal domain of the alpha subunit of RNA polymerase in LuxR-dependent transcriptional activation of the *lux* operon during quorum sensing. J*. Bacteriol.* **184**:4520-4528.

Fiori, M. (1992). A new bacterial disease of chrysanthemum: a stem rot by *Pseudomonas corrugata* Roberts et Scarlett. *Phytopathol. Med.* **31**, 110–114.

Fuqua, C., Greenberg, E.P. (2002). Listening in on bacteria: acyl-homoserine lactone signalling. *Nat. Rev. Mol. Cell Biol.* **3**, 685-695.

Fuqua, W.C., Winans, S.C., Greenberg, E.P. (1994). Quorum sensing in bacteria: The LuxR–LuxI family of cell density responsive transcriptional regulators. *J. Bacteriol.* **176**, 269-275.

Fuqua, W.C., Winans, S.C., Greenberg, E.P. (1996). Census and consensus in bacterial ecosystems: the LuxR–LuxI family of cell quorum-sensing regulators. *Annu Rev Microbiol* **50**: 727–751.

Fuqua, C., Parsek, M.R., Greenberg, E.P. (2001). Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. *Annu Rev Genet* **35**: 439–468.

Garbeva, P., Van Overbeek, L.S., Van Vuurde, J.W.L., Van Elsas, J.D. (2001). Analysis of endophytic bacterial communities of potato by plating and denaturing gradient gel electrophoresis (DGGE) of 16S rDNA based PCR fragments. *Microb. Ecol.* **41**, 369–383.

Gerard, J., Lloyd, R., Barsby, T., Haden, P., Kelly, M. T., Andersen, R. J. (1997). Massetolides A-H, antimycobacterial cyclic depsipeptides produced by two pseudomonads isolated from marine habitats. *J. Nat. Prod.* **60**:223-229.

Gewolb, J. (2002). Bioengineering. Working outside the protein-synthesis rules. *Science* **295**:2205-2207.

Graniti, A. (1991). Phytotoxins and their involvement in plant diseases. Introduction. *Experientia,* 47(8): 751-755.

Grgurina, I., and Benincasa, M. (1994). Evidence of the non ribosomal biosynthetic mechanism in the formation of syringomycin and syringopeptin,bioactive lipodepsipeptides of the phytopathogenic bacterium *Pseudomonas syringae* pv. *syringae. Ital. Biochem. Soc. Trans.* **5**:143.

Grilley, M. M., S. D. Stock, R. C. Dickson, R. L. Lester, and J. Y. Takemoto. 1998. Syringomycin action gene *SYR2* is essential for sphingolipid 4-hydroxylation in *Saccharomyces cerevisiae*. J. Biol. Chem. **273:**11062–11068.

Gross, H.; Stockwell, V.O.; Henkels, M.D.; Nowak-Thompson, B.; Loper, J.E.; Gerwick, W.H. (2007). The genomisotopic approach: a systematic method to isolate products of orphan biosynthetic gene clusters. *Chem. Biol.* **14**, 53–63.

Gross, H., and Loper, J. E. (2009) Genomics of secondary metabolite production by *Pseudomonas* spp. *Natural Product Reports* **26**, 1408-1446.
Gross, M., and Rudolph, K. (1987). Demonstration of levan and alginate in bean plants (*Phaseolus vulgaris*) infected by *Pseudomonas syringae* pv. *phaseolicola*. *Journal of Phytopathology*, **120**, 9-19.
Gustine, D.L., Sherwood, R.T., Lukezic, F.L., Moyer, B.G. (1990). Metabolites from *Pseudomonas corrugata* elicit phytoalexin biosynthesis in white clover. *Phytopathology*, **80**, 1427–1432.
Gustine, D.L., Sherwood, R.T., Lukezic, F.L., Moyer, B.G., Devlin, W.S. (1994). Metabolites of *Pseudomonas corrugata* elicit plant defense reactions. In: *Bioregulators and Natural Products* (Hedin, P.A., ed.), pp. 169–181. ACS Symposium Series. Washington, DC: Am. Chem. Soc.
Gustine, D.L., Sherwood, R.T., Moyer, B.G. (1995). Evidence for a new class of peptide elicitor of the hypersensitive reaction from the tomato pathogen *Pseudomonas corrugata.* Phytopathology. **85**, 848–853.
Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557-580.
Haas D. and Keel C. (2003). Regulation of antibiotic production in root-colonizing *Pseudomonas* spp. e relevance for biological control of plant disease. *Annu. Rev. Phytopathol.* **41**, 117-153.
Haas D. and Défago G. (2005). Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nature Reviews Microbiology* **3**:307–19.
Harshey RM (2003) Bacterial motility on a surface: many ways to a common goal. *Annu Rev Microbiol* **57**: 249–273.
Henrichsen J (1972) Bacterial surface translocation: a survey and a classification. *Bacteriol Rev* **36**: 478–503.
Henriksen, A., Anthoni, U., Nielsen, T. H., Sorensen, J., Christophersen, C., Gajhede, M. (2000). Cyclic lipoundecapeptide tensin from *Pseudomonas fluorescens* strain 96.578. *Acta Crystal.* **56**:113-115.
Herrero, M., de Lorenzo, V., and Timmis, K. N. (1990). Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertions of foreign genes in gram-negative bacteria. *J. Bacteriol.* **172**, 557-6567.
Hildebrand, P. D., Braun, P. G., McRae, K. B., Lu, X. (1998). Role of the biosurfactant viscosin in broccoli head rot caused by a pectolytic strain of *Pseudomonas fluorescens*. *Can. J. Plant Pathol.* **20**:296-303.
Hirano, S., and Upper, C.D. (2000). Bacteria in the leaf ecosystem with emphasis on *Pseudomonas syringae* – a pathogen, ice nucleus, and epiphyte. *Microbiology and Molecular Biology Review*s, **64,** 624-653.
Hoffmann, N., Rehm, B.H. (2004). Regulation of polyhydroxyalkanoate biosynthesis in *Pseudomonas putida* and *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* **237**:1–7.
Hoffmann, N., Rehm, B.H (2005), Nitrogen-dependent regulation of medium-chain length polyhydroxyalkanoate biosynthesis genes in pseudomonads. *Biotechnol Lett* **27**:279–282.
Hofte M. and De Vos P. (2006). Plant Pathogenic *Pseudomonas* species. *S.S.Gnanamanickam (ed.), Plant-Associated Bacteri*a, 507-533.
Howell C.R and Stipanovic R.D. (1979) Control of *Rhizoctonia solani* on cotton seedlings with *Pseudomonas fluorescens* and with an antibiotic produced by the bacterium. *Phytopathology* **69**: 480–482.
Howell, C.R. and Stipanovic, R.D. (1980). Suppression of *Pythium ultimum*-induced damping-off of cotton seedlings by *Pseudomonas fluorescens* and its antibiotic, pyoluteorin. *Phytopathology* **70**: 712–715.
Hutchison, M. L., Tester, M. A., Gross, D. C. (1995). Role of biosurfactant and ion channel-forming activities of syringomycin in transmembrane ion flux: A model for the mechanism of action in the plant-pathogen interaction. *Mol. Plant-Microbe Interact.* **8**:610-620.
Hutchison, M.L. and Gross,D. C. (1997). Lipopeptide phytotoxins produced by *Pseudomonas syringae* pv. *syringae*: comparison of the biosurfactant and ion channel-forming activities of syringopeptin and syringomycin. *Mol. Plant Microbe Interact.* **10**, 347–354.
Hutchison, M. L., and Johnstone, K. 1993. Evidence for the involvement of the surface- active properties of the extracellular toxin tolaasin in the manifestation of brown blotch disease symptoms by *Pseudomonas tolaasii* on *Agaricus bisporus. Physiol. Mol. Plant Pathol.* **42**:373-384
Iacobellis, N. S., P. Lavermicocca, I. Grgurina, M. Simmaco, and A. Ballio. 1992. Phytotoxic properties of *Pseudomonas syringae* pv. *syringae* toxins. *Physiol. Mol. Plant Pathol.* **40:**107–116.
Jayasinghe, S., Hristova, K. and White, S.H. (2001). Energetics, stability and prediction of transmembrane helices. *J Mol Biol* **312**, 927–934.
Juhas, M., Eberl, L., Tümmler, B. (2005). Quorum sensing: the power of cooperation in the world of *Pseudomonas*. *Environ. Microbiol.* **7**, 459-471.

Julmanop, C., Y. Takano, J. Y. Takemoto, and T. Miyakawa. 1993. Protection by sterols against the cytotoxicity of syringomycin in yeast *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* **139:**2323–2327.

Kadouri, D., Jurkevitch, E., Okon, Y., Castro-Sowinski, S. (2005). Ecological and agricultural significance of bacterial polyhydroxyalkanoates. *Crit. Rev. Microbiol.* **31**, 1–13.

Kang, H. and Gross, D. C. (2005). Characterization of a resistance-nodulation-cell division transporter system associated with the *syr-syp* genomic island of *Pseudomonas syringae* pv. *syringae*. *Appl. Environ. Microbiol.* **71**, 5056-5065.

Kaulin, Y. A., L. V. Schagina, S. M. Bezrukov, V. V. Malev, A. M. Feigin, J. Y. Takemoto,J. H. Teeter, and J. G. Brand. 1998. Cluster organization of ion channels formed by the antibiotic syringomycin E in bilayer lipid membranes. *Biophys. J.* **74:**2918–2925.

Kaulin, Y.A., Takemoto, J.Y., Schagina, L. V., Ostroumova, O.S., Wangspa, R., Teeter, J.H., and Brand, J.G. (2005). Sphingolipids influence the sensitivity of lipid bilayers to fungicide, syringomycin E. *J. Bioenerg. Biomembr.* 37, 339–348.

Kearns DB and Losick R (2003) Swarming motility in undomesticated *Bacillus subtilis. Mol Microbiol* **49**: 581–590.

Kersters, K., Ludwig, W., Vancanneyt, M., De Vos P., Gillis and Schleifer, K.H. (1996). Recent changes in the classification of the pseudomonads: an overview. *Syst. Appl. Microbiol.* **19**, 465–477.

Kessler, B. and Palleroni, N.J. (2000). Taxonomic implications of synthesis of poly-beta- hydroxybutyrate and other poly-beta-hydroxyalkanoates by aerobic pseudomonads. Int. J. Syst. Evol. Microbiol. **50**, 711–713.

Kim, J., Kim, J. G., Kang, Y., Jang, J. Y., Jog, G. J., Lim, J. Y., Kim, S., Suga, H., Nagamatsu, T., Hwang, I. (2004). Quorum sensing and the LysR-type transcriptional activator ToxR regulates toxoflavin biosynthesis and transport in *Burkholderia glumae*. *Mol. Microbiol.* **54**, 921–934.

Kinsinger RF, Shirk MC , Fall R (2003) Rapid surface motility in *Bacillus subtilis* is dependent on extracellular surfactin and potassium ion. *J Bacteriol* **185**: 5627–5631.

Kitten, T., Kinscherf, T. G., McEvoy, J. L., and Willis, D. K. 1998. A newly identified regulator is required for virulence and toxin production in *Pseudomonas syringae*. Mol. Microbiol. 28:917-929.

Koch, B., Nielsen, T. H., Sorensen, D., Andersen, J. B., Christophersen, C., Molin, S., Givskov, M., Sorensen, J., Nybroe, O. (2002). Lipopeptide production in *Pseudomonas* sp. strain DSS73 is regulated by components of sugar beet seed exudate via the Gac two-component regulatory system. *Appl. Environ. Microbiol.* **68**, 4509-4516.

Konnova, E. V., R. Hedman, C. J. Welch, B. Gerhardson. (2004). Character of massetolide A production by biocontrol strain *Pseudomonas* sp. MF-30, p. 565–568. *In* I. A. Tikhonovich, B. J. J. Lugtenberg, and N. A. Provorov (ed.), *Biology of plant-microbe interactions*, vol. 4. International Society for Plant-Microbe Interactions, St. Paul, MN.

Koumoutsi, A., Chen, X. H., Henne, A., Liesegang, H., Hitzeroth, G., Franke, P., Vater, J., Borriss, R. (2004). Structural and functional characterization of gene clusters directing nonribosomal synthesis of bioactive cyclic lipopeptides in *Bacillus amyloliquefaciens* strain FZB42. *J. Bacteriol.* **186**:1084-1096.

Kovacevich, P.A. and Ryder, M.H. (1991) Biocontrol performance characterization of *Pseudomonas corrugata* isolates PS 2140 and PS 2161. *Phytopathology*, **81**, 1178.

Kowall, M., Vater, J., Kluge, T., Stein, P., Ziessow, D. (1998). Separation and characterization of surfactin isoforms produced by Bacillus subtilis OKB 105. *Journal of Colloid and Interface Science*, **203**,1-8.

Kruijt M, Tran H & Raaijmakers JM (2009). Functional, genetic and chemical characterization of biosurfactants produced by plant growth-promoting *Pseudomonas putida* 267. J *Appl Microbiol* **107**: 546–556.

Kuiper, I., E. L. Lagendijk, R. Pickford, J. P. Derrick, G. E. M. Lamers, J. E. Thomas-Oates, B. J. Lugtenberg, G. V. Bloemberg. (2004). Characterization of two *Pseudomonas putida* lipopeptide biosurfactants, putisolvin I and II, which inhibit biofilm formation and break down existing biofilms. *Mol. Microbiol.* **51:**97–113.

Kumar, B., Trivedi, P., Pandey, A. (2007) *Pseudomonas corrugata*: A suitable bacterial inoculant for maize grown under rainfed conditions of Himalayan region. *Soil Biol and Biochiem* **39**: 3093-3100.

Lamont, I. L., Beare, P. A., Ochsner, U., Vasil, A. I., Vasil, M. L. (2002). Siderophore-mediated signaling regulates virulence factor production in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci* USA **99**,7072–7077.

Latoud C, Peypoux F,Michel G,(1990). Interaction of iturin A, a lipopeptide antibiotic, with *Saccharomyces cerevisiae* cells: influence of the sterol membrane composition. *Canadian Journal of Microbiology* **36**, 384–9.

Laursen, J. B. and Nielsen, J. (2004). Phenazine Natural Products: Biosynthesis, Synthetic Analogues, and Biological Activity. *Chem. Rev.* **104***,* 1663-1685.

Lavermicocca, P., Iacobellis, N. S., Simmaco, Graniti, A. (1997). Biological properties and spectrum of activity of *Pseudomonas syringae* pv. *syringae* toxins. *Physiol. Mol. Plant Pathol*. **50**:129-140.

Laville J., Blumer C., Von Schroetter C., Gaia V., Défago G., Keel C., Haas D. (1998). Characterization of the *hcnABC* gene cluster encoding hydrogen cyanide synthase and anaerobic regulation by ANR in the strictly aerobic biocontrol agent *Pseudomonas fluorescens* CHA0. *J. Bacteriol.* **180**: 3187–3196.

Lazzaroni, S., Bassarello, C., Bifulco, G., Lo Cantore, P., Evidente, A., Iacobellis N. S., Riccio, R., Gomez-Paloma, L. (2003). Chemical and biological characterisation of tolaasins A-E: New lipodepsipeptides produced by *Pseudomonas tolaasii*. Pages 245-254 in: *Pseudomonas syringae* Pathovars and Related Pathogens. N. S. Iacobellis, ed. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Lenz, R.W., Marchessault, R.H. (2005). Bacterial polyesters: biosynthesis, biodegradable plastics and biotechnology. *Biomacromolecules* **6**:1–8.

Lerat, E., Moran, N.A. (2004). The evolutionary history of quorum-sensing-systems in bacteria. *Mol. Biol. Evol*. **21**, 903-913.

Levi, C., and R. D. Durbin. (1986). The isolation and properties of a tabtoxin hydrolyzing aminopeptidase from the periplasm of *Pseudomonas syringae* pv. *tabaci*. *Physiol. Mol. Plant Pathol*. **28:** 345–352.

Licciardello, G., Bertani, I., Steindler, L., Bella, P., Venturi, V., Catara, V. (2007).*Pseudomonas corrugata* contains a conserved N-acyl homoserine lactone quorum sensing system; its role in tomato pathogenicity and tobacco hypersensitivity response. *FEMS Microbiol. Ecol*. 61, 222-234.

Licciardello, G., Bertani, I., Steindler, L., Bella, P., Venturi, V., Catara, V. (2009). The transcriptional activator *rfiA* is Quorum Sensing regulated by cotranscription with the *luxI* homolog *pcoI* and is essential for plant virulence in *Pseudomonas corrugata*. *Mol. Plant-Microbe Interact.* **22**,(12),1514-1522.

Licciardello G., Bella P., Catara, V. (2011). Quantitative detection of *Pseudomonas corrugata* and *Pseudomonas mediterranea* in tomato plants by duplex real-time PCR. *Journal of Plant Pathology*, **93** (3), 595-602.

Licciardello G., Strano C.P., Bertani I., Bella P., Fiore A., Fogliano V., Venturi V., Catara V. (2012). N-Acyl-homoserine lactones quorum sensing in tomato phytopathogenic *Pseudomonas* spp. is involved in lipodepsipeptide production. *J.Biotechnol.*, **159**, 274–282.

Lim, S.P., Roongsawang, N., Washio, K. and Morikawa, M. (2009). Flexible exportation mechanisms of arthrofactin in *Pseudomonas* sp. MIS38. *J. Appl. Microbiol*, 1-10.

Lindow, S.E., Arny, D.C., Upper, C.D. (1982) Bacterial ice nucleation: a factor in frost injury to plants. *Plant Physiol* **70**: 1084–1089.

Lindow, S. E., and Brandl, M. T. 2003. Microbiology of the phyllosphere. *Appl. Environ. Microbiol.* **69**:1875-1883.

Livak, K.J. and Schmittgen T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* **25**, 402–408.

Loper, J.E., Hassan, K.A., Davis, E.W. II, Lim, C.K., Mavrodi, D.V., Tetu, S.G., Radune, D., Hostetler, J., Brinkac, L.M., Durkin, A.S., Thomashow, L.S., Wechter, W.P., Kluepfel, D.A., Allen, A.E. and Paulsen, I.T. (2012). Direct Submission. The J. Craig Venter Institute, 9704 Medical Center Dr., Rockville, MD 20850, USA.

Lopez, M.M., Siverio, F., Albiach, R., Garcia, F. and Rodriguez, F.R. (1994). Characterization of Spanish isolates of *Pseudomonas corrugata* from tomato and pepper. *Plant Pathol*. **43**, 80–90.

Lops A (2005). Antimicrobial activity of secondary metabolites produced by different pathovars of *Pseudomonas syringae* and by strains of *P. avellanae*. *Phyopathol. Mediterr.* **44***,* 56–70.

Lu, S.E., Scholz-Schroeder, B.K., Gross, D.C. (2002). Characterization of the *salA*, *syrF*, and *syrG* regulatory genes located at the right border of the syringomycin gene cluster of *Pseudomonas syringae* pv. syringae*. Mol. Plant-Microbe Interact.* **15,** 43–53.

Maddula, V.S.R.K., Zhang, Z., Pierson, E.A., Pierson III, L.S. (2006). Quorum sensing and phenazines are involved in biofilm formation by *Pseudomonas chlororaphis* (*aureofaciens*) strain 30-84. *Microbial Ecology* **52**, 289-301.

Magazin, M.D., Moores, J.C., Leong, J. (1986). Cloning of the gene coding for the outer membrane receptor protein for ferric pseudobactin, a siderophore from a plant growth-promoting. *Pseudomonas* strain. *J. Biol. Chem.* **261**, 795-799.

Magyarosy, A.C. and Buchanan, B.B. (1995). First report of *Pseudomonas corrugata* causing pith necrosis on geraniums. *Phytopathology*, **85**, 1040.

Mandel JR, Nambeesan S, Bowers JE, Marek LF, Ebert D, et al. (2013) Association Mapping and the Genomic Consequences of Selection in Sunflower. *PLoS Genet* 9(3): e1003378. doi:10.1371/journal.pgen.1003378.

Mansfield J, Genin S, Magori S, Citovsky V, SriariyanumM, Ronald P, et al (2012). Top 10 plant pathogenic bacteria in molecular plant pathology. *Mol Plant Pathol* ;13: 614–29.

Marahiel, M. A., Stachelhaus, T., Mootz H. D. (1997). Modular peptide synthetases involved in nonribosomal peptide synthesis. *Chem. Rev.* **97**, 2651-2673.

Mark, G.L., Morrissey, J.P., Higgins, P. and O'Gara, F. (2006) Molecular based strategies to exploit *Pseudomonas* biocontrol strains for environmental biotechnology applications. *FEMS Microbiol. Ecol.* **56**, 167–177.

Martinelli, D., Grossmann, G., Séquin, U., Brandl, H., and Bachofen, R. (2004). Effects of natural and chemically synthesized furanones on quorum sensing in *Chromobacterium violaceum*. *BMC Microbiol.* **4**, 25.

Matas, I.M., Pérez-Martínez, I., Quesada, J.M., Rodriguez-Hervá, J.J., Penyalver, R., and Ramos, C. (2009). *Pseudomonas savastanoi* pv. savastanoi contains two *iaaL* paralogs, one of which exhibits a variable number of a trinucleotide (TAC) tandem repeat. *Appl Environ Microbiol* **75:** 1030–1035.

Matthijs, S., Baysse, C., Koedam, N., Tehrani, A.K., Verheyden, L., Budzikiewicz, H., Schäfer, M., Hoorelbeke, B., Meyer, JM., De Greve, H., Cornelis, P. (2004) The *Pseudomonas* siderophore quinolobactin is synthesized from xanthurenic acid, an intermediate of the kynurenine pathway. *Molecular Microbiology* **52** (2): 371-384.

Matthijs, S., Budzikiewiczb, H., Schäferb, M., d Watheletc, B., Cornelis P. (2008). Ornicorrugatin, a new siderophore from *Pseudomonas fluorescens* AF76. *Z. Naturforsch.* **63c**, 8-12.

Maurhofer, M.; Keel, C.; Schnider, U.; Voisard, C.; Haas, D. & Defago, G. (1992). Influence of enhanced antibiotic production in *Pseudomonas fluorescens* strain CHA0 on its disease suppressive capacity. *Phytopathology*, **82**(2), 190-195.

Malev, V.V., Schagina, L.V., Gurnev, P.A., Takemoto,J.Y., Nestorovich, E. M., and Bezrukov, S.M. (2002). Syringomycin E channel: a lipidic pore stabilized by lipopeptide? *Bio-phys.J.* **82,** 1985–1994.

Mavrodi D.V, Tomashow L.S. (2006) Phenazine compounds in fluorescent *Pseudomonas* spp. biosynthesis and regulation. *Annual review of Phytopathology* **44**: 417– 445.

Mazzola, M., Zhao, X., Cohen, M.F., Raaijmakers, J.M., (2007). Cyclic lipopeptide surfactant production by *Pseudomonas fluorescens* is not required for suppression of complex Pythium spp. Populations. Phytopathology 97, 1348– 1355.

McCarter L & Silverman M (1990) Surface-induced swarmer cell differentiation of Vibrio parahaemolyticus. *Mol Microbiol* **4**: 1057–1062.

McCullagh, M., Utkhede, R., Menzies, J.G., Punja, Z.K. and Paulitz, T.C. (1996). Evaluation of plant growth-promoting rhizobacteria for biological control of Pythium root rot of cucumbers grown in rockwool and effects on yield. *Eur. J. Plant Pathol.* **102**, 747–755.

McClean, K. H., Winson, M. K., Fish, L., Taylor, A., Chhabra, S. R., Camara, M., Daykin, M., Lamb, J. H., Swift, S., Bycroft, B. W., Stewart, G. S. A. B., Williams, P. (1997). Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. *Microbiology* **143**, 3703-3711.

Melotto, M., Underwood, W., Koczan, J., Nomura, K., He, S.Y. (2006) Plant stomata function in innate immunity against bacterial invasion. *Cell* **126**: 969-980.

Méndez, C. and Salas, J.A. (2001). The role of ABC transporters in antibiotic-producing organisms: drug secretion and resistance mechanisms. *Res Microbiol* **152**, 341–350.

Menestrina G., Coraiola M., Fogliano V., Fiore A., Grgurina I., Carpaneto A., Gambale F., Dalla Serra M. (2003). Antimicrobial lipodepsipeptides from *Pseudomonas* spp: a comparison of their activity on model membranes, in: S.N. Iacobellis (Ed.), *Pseudomonas syringae* and Related Pathogens. *Biology and Genetic, Kluwer Academic Publishers, Dordrecht,* NL, pp. 185–198.

Meyer, J.M., Geoffroy, V.A., Baida, N., Gardan, L., Izard, D., Lemanceau, P., Achouak, W., Palleroni, N.J. (2002). Siderophore typing, a powerful tool for the identification of fluorescent and nonfluorescent pseudomonads. *Appl. Environ. Microbiol.* **68**, 2745–2753.

Michelsen C. F., Stougaard P., 2012. Hydrogen cyanide synthesis and antifungal activity of the biocontrol strain *Pseudomonas fluorescens* In5 from Greenland is highly dependent on growth medium. Can. J. Microbiol. 58: 381–390.

Miller, M.B. and Bassler, B.L. (2001). Quorum sensing in bacteria. *Annu Rev Microbiol* **55**: 165–99.

Miller, J. H. (1972). Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Misaghi I, and Grogan RC (1969) Nutritional and biochemical comparisons of plant-pathogenic and saprophytic fluorescent pseudomonads. Phytopathology 59: 1436–1450.

Mitchell, R. E. (1976). Isolation and structure of a chlorosis-inducing toxin of *Pseudomonas phaseolicola*. *Phytochemistry* **15:** 1941–1947.

Mitchell, R. E. (1982). Coronatine production by some phytopathogenic pseudomonads. *Physiol. Plant Pathol.* **20:**83–89.

Mitchell, R. E., and Bieleski, R. L. (1977). Involvement of phaseolotoxin in halo blight of beans. Transport and conversion to functional toxin. *Plant Physiol.* **60:**723–729.

Mitchell, R. E., Hale, C. N., Shanks, J. C. (1983). Production of different pathogenic symptoms and different toxins by strains of *Pseudomonas syringae* pv. *tomato* not distinguishable by gel-immunodiffusion assay. Physiol. Plant Pathol. **23:**315–322.

Monti, S. M., Gallo, M., Ferracane, R., Borrelli, R. C., Ritieni, A., Greco, M. L., Graniti, A., Fogliano, V. (2001). Analysis of bacterial lipodepsipeptides by matrix-assisted laser desorption/ionisation time-of-flight and high-performance liquid chromatography with electrospray mass spectrometry. *Rapid Commun. Mass Spectrom.* **15**:623-628.

Moore, R. A., A. N. Starratt, S.-W. Ma, V. L. Morris, D. A. Cuppels. (1989). Identification of a chromosomal region required for biosynthesis of the phytotoxin coronatine by *Pseudomonas syringae* pv. *tomato*. *Can. J. Microbiol.* **35:** 910–917.

Moore, E.R.B., Mau, M., Arnscheidt, A., Böttger, E.C., Hutson, R.A., Collins, M.D., Van de Peer, Y., De Wachter, R. and Timmis, K.N. (1996). The determination and comparison of the 16S rRNA gene sequence of species of the genus *Pseudomonas (sensu stricto)* and estimation of the natural intrageneric relationship. *Syst. Appl. Microbiol.* **19**, 478–492.

Morikawa, M., Daido, H., Takao, T., Murata, S., Shimonishi, Y., Imanaka, T. (1993). A new lipopeptide biosurfactant produced by *Arthrobacter* sp. strain MIS38. *J. Bacteriol.* **175**, 6459– 6466.

Mott, K. A., and J. Y. Takemoto. 1989. Syringomycin, a bacterial phytotoxin, closes stomata. *Plant Physiol.* **90:**1435–1439.

Moura, M.L., Jacques, M.A., Brito, L.M., Mourão, I.M., Duclos, J. (2005). Tomato pith necrosis (TPN) caused by P. corrugata and P. mediterranea: severity of damages and crop loss assessment. *Acta Hort.* (ISHS), **695**, 365–372.

Moynihan, J.A., Morrissey, J.P., Coppoolse, E.R., Stiekema, W.J., O'Gara, F., Boyd, E.F. (2009) Evolutionary history of the *phl* gene cluster in the plant-associated bacterium *Pseudomonas fluorescens*. *Appl Environ Microbiol* **75**: 2122–2131.

Mulet, M, Lalucat, J., García-Valdés, E. (2010). DNA sequence-based analysis of the *Pseudomonas* species. *Environ. Microbiol.* **12**, 1513-30.

Muller P., Schiller S., Wieprecht T., Dathe M., Herrmann A. (2000). Continuous measurement of rapid transbilayer movement of a pyrene-labeled phospholipid analogue. *Chem. Phys. Lipids* **106**:89-99.

Neu, T. R. 1996. Significance of bacterial surface-active compounds in interaction of bacteria with interfaces. *Microbiol. Rev.* **60**:151-166.

Nielsen, T. H., Christophersen, C., Anthoni, U., Sorensen, J. (1999). Viscosinamide, a new cyclic depsipeptide with surfactant and antifungal properties produced by *Pseudomonas fluorescens* DR54. *J. Appl. Microbiol.* **87**:80-90.

Nielsen, T. H., Sorensen, D., Tobiasen, C., Andersen, J. B., Christophersen, C., Givskov, M., Sorensen, J. (2002). Antibiotic and biosurfactant properties of cyclic lipopeptides produced by fluorescent *Pseudomonas* spp. from the sugar beet rhizosphere. *Appl. Environ. Microbiol.* **68**:3416-3423.

Nybroe O. and Sørensen J. (2004) Production of cyclic lipopeptides by fluorescent pseudomonads. *Pseudomonas*, Biosynthesis of Macromolecules and Molecular Metabolism (Ramos J-L, ed), Kluwer Academic/Plenum Publishers, New York. pp. 147–172.

O'Brien, H.E., Thakur, S., Gong, Y., Fung, P., Zhang, J., Yuan, L., Wang, P. W., Yong, C., Scortichini M., and Guttman, D. S. (2012). Extensive remodeling of the *Pseudomonas syringae* pv. *avellanae* type III secretome associated with twoindependent host shifts onto hazelnut. *BMC Microbiology*, **12**,141.

Ongena, M. and Jacques P. (2008) *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. *Trends Microbiol* **16**: 115–125.

Palleroni, N.J. (1984) Genus *Pseudomonas*. In *Bergey's Manual of Systematic Bacteriology*, Vol. 1. Krieg, N.R., and Holt, J.G. (eds). Baltimore, MD, USA: Williams & Wilkins, pp. 141–199

Palmeri, R., Pappalardo, F., Fragalà, M., Tomasello, M., Damigella, A., Catara A.F. (2012). Polyhydroxyalkanoates (PHAs) Production Through Conversion of Glycerol by Selected Strains of *Pseudomonas mediterranea* and *Pseudomonas corrugata. The Italian Association of Chemical Engineering,* (**27**), 121-126.

Pandey, A., and Palni, L.M.S. (1998) Isolation of *Pseudomonas corrugata* from Sikkim Himalaya. World J. *Microbiol. Biotechnol.*;14:411–3.

Pandey, A., Palni, L.M.S. and Hebbar, K.P. (2001). Suppression of dampingoff in maize seedlings by *Pseudomonas corrugata*. *Microbiol. Res.* **156**, 191–194.

Parkinson, J.S. and Kofoid, E.C. (1992). Communication modules in bacterial signalling proteins. *Annu. Rev. Genet*. **26**:71-112.

Patel, H.K., Passos, D., Devescovi, G., Maraite, H., Paszkiewicz, K., Studholme, D.J. and Venturi,V. Draft genome sequence of *Pseudomonas fuscovaginae*, a broad host-range phytopathogen. Unpublished.

Paulitz, T., Zhou, C. and Rankin, L. (1992) Selection of rhizosphere bacteria for biological control of Pythium aphanidermatum on hydroponicallygrown cucumber. *Biol. Control*, **2**, 226–237.

Paulsen, I.T., Press, C., Ravel, J., Kobayashi, D., Myers, G.S., Mavrodi, D., DeBoy, R.T., Seshadri, R., Ren, Q., Madupu, R., Dodson, R.J., Durkin, S., Brinkac, L.M., Daugherty, S.C., Sullivan, S.A., Rosovitz, M., Gwinn, M.L., Zhou, L., Nelson, W.C., Weidman, J., Watkins, K., Tran, K., Khouri, H.M., Pierson, E., Pierson, L. III, Thomashow, L. and Loper, J. (2005). Complete genome sequence of the plant commensal *Pseudomonas fluorescens* Pf-5. *Nature Biotech* **23** (7), 873-878.

Pekhtereva, E.S., Kornev, K.P., Matveeva, E.V., Polityko, V.A., Budenkov, N.I., Ignatov, A.N., Schaad, N.W. (2009). Pith necrosis of tomato in Russia. *Acta Hort*. (ISHS) **808**, 251-254.

Peñaloza-Vazquez, A., Kidambi, S.P., Chakrabarty, A.M., & Bender, C. (1997). Characterization of the alginate biosynthetic gene cluster in *Pseudomonas syringae* pv. *syringae*. *Journal of Bacteriology*, **179**, 4464-4472.

Pesci, E.C. and Iglewski, B.H. (1997). The chain of command in *Pseudomonas* quorum sensing. *Trends Microbiol* **5**:132–34.

Pessi G. and Haas D. (2000). Transcriptional control of the hydrogen cyanide biosynthetic genes *hcnABC* by the anaerobic regulator ANR and the quorum-sensing regulators LasR and RhlR in *Pseudomonas aeruginosa*. *J. Bacteriol*. **182**: 6940-6949.

Piper, K.R., Beck von Bodman, S., Farrand, S. (1993). Conjugation factor of *Agrobacterium tumefaciens* regulates Ti plasmid transfer by autoinduction. *Nature* **362**, 448-450.

Quigley, N.B., Mo, Y.Y., Gross, D.C. (1993). SyrD is required for syringomycin production by *Pseudomonas syringae* pathovar *syringae* and is related to a family of ATP-binding secretion proteins. *Mol Microbiol* Aug; **9**(4), 787-801.

Quinones, B., Pujol, C. J., Lindow, S. E. (2004). Regulation of AHL production and its contribution to epiphytic fitness in *Pseudomonas syringae*. *Mol. Plant-Microbe Interact*. **17**:521-531.

Quinones, B., Dulla, G., Lindow, S.E. (2005). Quorum sensing regulates exopolysaccharide production, motility, and virulence in *Pseudomonas syringae*. *Mol. Plant-Microbe Interact.* 18:682-693

Raaijmakers J.M., Weller D.M., Thomashow L.S. (1997) Frequency of antibiotic-producing *Pseudomonas* spp. in natural environments. *Appl Environ Microb* **63**: 881–887.

Raaijmakers, J.M., De Bruijn, I., De Kock, M.J.D. (2006). Cyclic lipopeptide production by plant associated *Pseudomonas* spp.: diversity, activity, biosynthesis, and regulation. *Mol. Plant-Microbe Interact*. **19**, 699–710.

Raaijmakers, J. M., de Bruijn, I., Nybroe, O., Ongena M. (2010). Natural functions of lipopeptides from *Bacillus* and *Pseudomonas* more than surfactants and antibiotics. *FEMS Microbiol Rev,* **34**, 1037–1062.

Raaijmakers J. M. and Mazzola (2012)**.** Diversity and Natural Functions of Antibiotics Produced by Beneficial and Plant Pathogenic Bacteria *Annu. Rev. Phytopathol*. **50**: 403–24.

Rainey, P. B., Brodey, C. L., and Johnstone, K. 1991. Biological properties and spectrum of activity of tolaasin, a lipodepsipeptide toxin produced by the mushroom pathogen *Pseudomonas tolaasii*. *Physiol. Mol. Plant Pathol*. **39**:57-70.

Ramette, A., Frapolli, M., Défago, G., Moënne-Loccoz, Y. (2003). Phylogeny of HCN synthase-encoding *hcnBC* genes in biocontrol fluorescent *Pseudomonas* and its relationship with host plant species and HCN synthesis ability. *Mol. Plant-Microbe Interact*. **16**, 525–535.

Ravel, J. and Cornelis, P. (2003). Genomics of pyoverdine-mediated iron uptake in pseudomonads. *Trends Microbiol* **11**, 195–200.

Rezzonico, F., Zala, M., Keel, C., Duffy, B., Moënne-Loccoz, Y., Défago, G. (2007) Is the ability of biocontrol fluorescent pseudomonads to produce the antifungal metabolite 2,4-diacetylphloroglucinol really synonymous with higher plant protection? *New Phytol.* **173**(4):861-72.

Risse D., Beiderbeck H., Taraz K., Budzikiewicz H., Gustine D. (1998), Corrugatin, a lipopeptide from *Pseudomonas corrugata*. Z. *Naturforsch*. **53c**, 295D 304.

Rodrigues, L. M. R., Destéfano, S. A. L., Beriam, L. O. S., Rodrigues-Neto, J. (2010). Occurrence of *Pseudomonas mediterranea* on tomato in Brazil. *Plant Pathol*, **59**, 797.

Rokni-Zadeh, H.a Li, W., Sanchez-Rodriguez, A., Sinnaeve, D., Rozenski, J., Martins, J.C., De Mota, R. (2012). Genetic and Functional Characterization of Cyclic Lipopeptide White-Line-Inducing Principle (WLIP) Production by Rice Rhizosphere Isolate *Pseudomonas putida* RW10S2. *Appl. Environ. Microbiol.* **78**(14):4826-4834.

Roongsawang, N., Hase, K., Haruki, M., Imanaka, T., Morikawa, M., Kanaya, S. (2003). Cloning and characterization of the gene cluster encoding arthrofactin synthetase from *Pseudomonas* sp. MIS38. *Chem Biol* **10**, 869–880.

Roongsawang, N., Washio, K., Morikawa, M. (2011). Diversity of Nonribosomal Peptide Synthetases Involved in the Biosynthesis of Lipopeptide Biosurfactants. *Int. J. Mol. Sci.* **12**: 141-172.

Rudolph, K.W.E., Gross, M., Neugebauer, M., Hokawat, S., Zachowski, A., Wydra, K. et al. (1989). Extracellar polysaccharides as determinants of leaf spot diseases caused by pseudomonads and xanthomonads. In Graniti, A. Durbin, R.D. & Ballio, A. A. (Eds). *Phytotoxins and Plant Pathogenesis* (pp. 177-218). Berlin: Springer.

Ryder, M.H. and Borrett, M.A. (1991). Root colonization by non-fluorescent Pseudomonads used for the control of wheat take-all. *Bull. SROP*, **14**, 302–307.

Ryder, M.H. and Rovira, A.D. (1993). Biological control of take-all of glasshouse-grown wheat using strains of *Pseudomonas corrugata* isolated from wheat field soil. *Soil Biol. Biochem.* **25**, 311–320.

Ryder, M.H., Ophel-Keller, K.M., Dhindsa, H., Veal, D.A. (1994). Detection and characterization of a genetically modified biocontrol bacterium in field soil. In: Improving Plant Productivity with Rhizosphere Bacteria (Ryder, M.H., Stephens, P.M. and Bowen, G.D., eds), pp. 261–263. *Adelaide*: CSIRO.

Ryder, M.H., Yan, Z., Terrace, T.E., Rovira, A.D., Tang, W.H., Correll, R.L. (1999). Use of strains of *Bacillus* isolated in China to suppress take-all and Rhizoctonia root rot, and promote seedling growth of glasshouse-grown wheat in Australian soils. *Soil Biol. Biochem*. **31**, 19–29.

Sahin, F., Aysan, Y., Saygili, H. (2005). First observation of pith necrosis on tomato caused by some *Pseudomonas* species in Turkey. *Acta Hortic*. **695**, 93–96.

Saier, M. H., Jr,, and Paulsen, I. T. (2001). Phylogeny of multidrug transporters. *Semin. Cell. Dev. Biol.* **12**:205-213.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. USA.

Scaloni, A., Dalla Serra, M., Amodeo, P., Mannina, L., Vitale, R. M., Segre, A. L., Cruciani, O., Lodovichetti, F., Greco, M. L., Fiore, A.,Gallo, M., D'Ambrosio, C., Coraiola, M., Menestrina, G., Granit, A.,and Fogliano, V. (2004). Structure, conformation and biological activity of a novel lipodepsipeptide from *Pseudomonas corrugata*: Cormycin A. *Biochem. J.* **384**, 25-36.

Scarlett, C.M., Fletcher, J.T., Roberts, P. and Lelliott, R.A. (1978). Tomato pith necrosis caused by *Pseudomonas corrugata*. n. sp. *Ann. Appl. Biol*. **88**,105-114.

Schmidt, C.S., Agostini, F., Leifert, C., Killham, K., Mullins, C.E. (2004). Influence of inoculum density of the antagonistic bacteria *Pseudomonas fluorescens* and *Pseudomonas corrugata* on sugar beet seedling colonisation and suppression of Pythium damping off. Plant Soil. **265**, 111–122.

Schisler, D.A. and Slininger, P.J. (1994). Selection and performance of bacterial strains for biologically controlling Fusarium dry rot of potatoes incited by *Gibberella pulicaris*. *Plant Dis.* **78**, 251–255.

Schlegel K., **Taraz K.**, **Budzikiewicz H.** (2004). The stereoisomers of pyochelin, a siderophore of *Pseudomonas aeruginosa*. *Biometals* **17**:409–414

Schneider, E. and Hunke, S. (1998). ATP-binding-cassette (ABC) transport systems: Functional and structural aspects of the ATP-hydrolyzing subunits / domains. *FEMS Microbiol Rev* **22**, 1–20.

Scholz-Schroeder, B. K., Hutchison, M. L., Grgurina, I., Gross, D. C. (2001). The contribution of syringopeptin and syringomycin to virulence of *Pseudomonas syringae* pv. *syringae* strain B301D on the basis of *sypA* and *syrB1* biosynthesis mutant analysis. *Mol. Plant-Microbe Interact.* **14,** 336–348.

Scholz-Schroeder, B.K., Soule, J.D., Gross, D.C. (2003). The *sypA*, *sypB*, and *sypC* synthetase genes encode twenty-two modules involved in the nonribosomal peptide synthesis of syringopeptin by *Pseudomonas syringae* pv. *syringae* B301D. *Mol. Plant Microbe Interact.* **16** (4), 271-280.

Scortichini, M. (1989). Occurrence in soil and primary infections of *Pseudomonas corrugata* Roberts and Scarlett. *J. Phytopathol.* **125**, 33–40.

Silby, M.W., Cerdeno-Tarraga, A.M., Vernikos, G.S., Giddens, S.R., Jackson,R.W., Preston, G.M., Zhang, X.X., Moon, C.D., Gehrig, S.M., Godfrey, S.A., Knight, C.G., Malone, J.G., Robinson, Z., Spiers, A.J., Harris, S., Challis, G.L., Yaxley, A.M., Harris, D., Seeger, K., Murphy, L., Rutter, S., Squares, R., Quail, M.A., Saunders, E., Mavromatis, K., Brettin, T.S., Bentley, S.D., Hothersall, J., Stephens, E., Thomas, C.M., Parkhill, J., Levy, S.B., Rainey, P.B., Thomson, N.R. (2009). Genomic and genetic analyses of diversity and plant interactions of *Pseudomonas fluorescens*. *Genome Biol.* **10** (5), R51.

Silby MW, Winstanley C, Godfrey SAC, Levy SB, Jackson RW (2011) *Pseudomonas* genomes: diverse and adaptable. *FEMS Microbiol Rev* **35**: 652–680.

Shaw, P. D., Ping, G., Daly, S. L., Cha, C., Cronan, Jr.,J. E., Rinehart, K. L., Farrand, S. K. (1997). Detecting and characterizing N-acyl-homoserine lactone signal molecules by thin-layer chromatography. *Proc. Natl. Acad. Sci.* USA. **94**, 6036-6041.

Sieber, S. A., and Marahiel, M. A.(2005). Molecular mechanisms underlying nonribosomal peptide synthesis: Approaches to new antibiotics. *Chem. Rev.* **105**:715-738.

Simon, R, Priefer, U., Puler, A. (1983). A broad host range mobilization system for *in vivo* genetic engineering. *Biotechnology* **1,** 784–791.

Siverio, F., Cambra, M., Gorris, M.T., Corzo, J., Lopez, M.M. (1993). Lypopolysaccharides as determinant of serological variability in *Pseudomonas corrugata. Appl. Environ. Microbiol.* **59**, 1805–1812.

Slavov, S. (2005). Phytotoxins and in vitro screening for improved disease resistant plants.*Biotechnol. & Biotechnol.* Eq. 19/2005 20th Anniversary AgroBioInstitute - R&D Special Issue.

Snell, K.D. and Peoples, O.P. (2002). Polyhydroxyalkanoate polymers and their production in transgeneic plants. *Metab Eng* **4**:29–40.

Solaiman, D.K.Y., Ashby, R.D., Foglia, T.A. (2000). Rapid and specific identification of medium-chain-length polyhydroxyalkanoate synthase gene by polymerase chain reaction. *Appl. Microbiol. Biotechnol.* **53**, 690–694.

Solaiman, D.K., Catara, V., Greco, S. (2005). Poly (hydroxyalkanoate) synthase genotype and PHA production of *Pseudomonas corrugata and P. mediterranea. J. Ind. Microbiol. Biotechnol.* **32**, 75–82.

Solaiman, D., Ashby, R.D., Hotchkiss, A.T., Foglia, T.A. (2006). Biosynthesis of medium-chain-length poly (hydroxyalkanoates) from soy molasses. *Biotechnol. Lett.* **28**, 157–162.

Sørensen KN, Kim KH & Takemoto JY (1998) PCR detection of cyclic lipodepsinonapeptide-producing *Pseudomonas syringae* pv. *syringae* and similarity of strains. *Appl Environ Microb* **64**: 226–230.

Sorensen, D., Nielsen, T. H., Christophersen, C., Sorensen, J., Gajhede, M. (2001). Cyclic lipoundecapeptide amphisin from *Pseudomonas* sp. strain DSS73. *Acta Crystallogr.* **57**:1123-1124.

Spaink, P. H., Okker, R. J. H., Wijffelman, C. A., Pees, E., Lugtenberg, B. J. J. (1987). Promoters in the nodulation region of the *Rhizobium leguminosarum* Sym plasmid pRL1JI. *Plant Mol. Biol.* **9,** 27–39.

Stachel, S., An, G., Flores, C., Nester, E. (1985). A Tn3 *lacZ* transposon for the random generation of-galactosidase gene fusions: application to the analysis of gene expression in *Agrobacterium*. *EMBO J.* **4**, 891–898.

Stanier RY, Palleroni NJ & Doudoroff M (1966) The aerobic pseudomonads: a taxonomic study. *J Gen Microbiol* **43**: 159–271.

Steindler L, and Venturi V (2007) Detection of quorum-sensing *N*-acyl homoserine lactone signal molecules by bacterial biosensors. *FEMS Microbiol. Lett.* **266**: 1-9

Stevens, A.M. and Greenberg, E.P. (1997). Quorum sensing in *Vibrio fischeri*: essential elements for activation of the luminescence genes. *J Bacteriol* **179**: 557–62.

Stewart PS & Franklin MJ (2008) Physiological heterogeneity in biofilms. *Nat Rev Microbiol* **6**: 199–210.

Stover CK, Pham XQ, Erwin AL *et al.* (2000) Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. *Nature* **406**: 959–964.

Surico, G., Iacobellis, N.S., and Sisto, A. (1985). Studies on the role of indole-3-acetic acid and cytokinins in the formation of knots on olive and oleander plants by *Pseudomonas syringae* pv. *savastanoi*. *Physiol Plant Pathol* **26:** 309–320.

Surico, G., Lavermicocca, P. and Iacobellis, N. S. (1998). Produzione di siringomicina e siringotossina in colture di *Pseudomonas syringae* pv. *syringae*. *Phytopathol. mediterr.* **27**, 163-168.

Sutra, L., Siverio, F., Lopez, M.M., Hanaul,t G., Bollet, C. and Gardan, L. (1997). Taxonomy of *Pseudomonas* strains isolated from tomato pith necrosis: emended description of *Pseudomonas corrugata* and proposal of three unnamed fluorescent *Pseudomonas* genomo-species. *Int J System Bacteriol.* 47, 1020-1033.

Szabo, Z., Budai, M. ,Blasko ,K., and Grof, P. (2004). Molecular dynamics of the cyclic lipodepsipeptides' action on model membranes: effects of syringopeptin 22A, syringomycin E, and syringotoxin studied by EPR technique. *Biochim. Biophys. Acta* 1660, 118–130.

Swift ,S., Winson, M. K., Chan, P. F., Bainton, N. J., Birdsall, M., Reeves, P. J., Rees, C. E. D., Throup, J.P., Bycroft, B.W., Salmond, J.P.C., Williams, P., Stewart, G.S.A. (1993). A novel strategy for the isolation of *luxI* homologues: evidence for the widespread distribution of a LuxR: LuxI superfamily in enteric bacteria. *Mol. Microbiol.* **10**, 511–520.

Taguchi, N., Y. Takano, C. Julmanop, Y. Wang, S. Stock, J. Takemoto, and T. Miyakawa. 1994. Identification and analysis of the *Saccharomyces cerevisiae* SYR1 gene reveals that ergosterol is involved in the action of syringomycin. *Microbiology* **140:**353–359.

Taguchi, F., Ogawa, Y., Takeuchi, K., Suzuki, T., Toyoda, K., Shiraishi,T., Ichinose, Y. (2006). A homologue of the 3-oxoacyl-(acyl carrier protein) synthase III gene located in the glycosylation island of *Pseudomonas syringae* pv. *tabaci* regulates virulence factors via *N*-acyl homoserine lactone and fatty acid synthesis. *J. Bacteriol.* **188**, 8376–8384.

Tarkowski P and Vereecke V. (2013). Threats and opportunities of plant pathogenic bacteria. *Biotechnol Adv*, http:// dx.doi.org/10.1016/j.biotechadv.2013.11.001.

Takeda, R. (1958). Pseudomollas pigments L. Pyroluteorin. a new chlorine-containing pigment produced by *Pseudomonas aeruginosa*. *Hakko Kogsku Zasschi* **36**: 281.

Takemoto, J.Y. ,Brand, J.G., Kaulin, Y.A., Malev, V.V., Schagina, L. V. ,and Blasko, K. (2003). "The syringomycins: lipodepsipeptide pore formers from plant bacterium. *Pseudomonas syringae*," in *Pore Forming Peptides and ProteinToxins*, eds G. Menestrina, M. Dalla Serra, and P.Lazarovici (London: TaylorandFrancis), 260–271.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28** (10), 2731–2739.

Thomashow, L.S. and Weller, D.M. (1988). Role of a phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminis* var. *tritici*. *J Bacteriol* ,**170**: 3499–3508.

Thrane C, Harder Nielsen T, Neiendam Nielsen M, Sørensen J & Olsson S (2000) Viscosinamide-producing *Pseudomonas fluorescens* DR54 exerts a biocontrol effect on Pythium ultimum in sugar beet rhizosphere. *FEMS Microbiol Ecol* **33**: 139–146.

Tran H, Ficke A, Asiimwe T, Hofte M & Raaijmakers JM (2007) Role of the cyclic lipopeptide massetolide A in biological control of Phytophthora infestans and in colonization of tomato plants by Pseudomonas fluorescens. *New Phytol* **175**: 731– 742.

Tripathi, R. K., and D. Gottlieb. (1969). Mechanism of action of the antifungal antibiotic pyrrolnitrin. *J. Bacteriol.* **100**: 310-318.

Trivedi P., Pandey A., Palni L. M. S. (2008). *In vitro* evaluation of antagonistic properties of *Pseudomonas corrugata. Microbiological* Research **163**: 329—336.

Tseng, C. F., Burger, A. Mislin, G. L., Schalk, I. J., Yu, S. S., Chan, S. I. Abdallah, M. A. (2006). Bacterial siderophores: the solution stoichiometry and coordinationof the Fe(III) complexes of pyochelin and related compounds. *J Biol Inorg Chem* **11**: 419–432.

Uchytil, T. F., and R. D. Durbin. (1980). Hydrolysis of tabtoxins by plant and bacterial enzymes. *Experientia* **36:** 301–302.

Uppalapati S.R., Ayoubi, P., Weng, H., Palmer, D.A., Mitchell, R.E., Jones, W., Bender, C.L. (2005) The 1105 phytotoxin coronatine and methyl jasmonate impact multiple phytohormone pathways in tomato. *Plant J* 1106 **42**: 201-217.

Vallet-Gely, I., Novikov, A., Augusto, L., Liehl, P., Bolbach, G., Pe´chy-Tarr, M.,Cosson, P., Keel, C., Caroff, M. and Lemaitre, B. (2010). Association of hemolytic activity of *Pseudomonas entomophila*, a versatile soil bacterium, with cyclic lipopeptide production. *Appl. Environ. Microbiol.* **73** (3), 910–921.

Van de Mortel JE, Tran H, Govers F & Raaijmakers JM (2009) Cellular responses of the late blight pathogen Phytophthora infestans to cyclic lipopeptide surfactants and their dependence on G proteins. *Appl Environ Microb* **75**: 4950–4957.

Venturi, V. (2006). Regulation of quorum sensing in *Pseudomonas*. *FEMS Microbiol. Rev.* **30**, 274–291.

Vidaver, A.K. and P.A. Lambrecht (2004). Bacteria as plant pathogens. *The Plant Health Instructor*. DOI: 10.1094/PHI-I-2004-0809-01

Vincent J. M., (1947). Distortion of fungal hyphae in the presence of certain inhibitors. *Nature* **150**:850.

Visca, P., Leoni, L., Wilson, M. J. & Lamont, I. L. (2002). Iron transport and regulation, cell signalling and genomics: lessons from *Escherichia coli* and *Pseudomonas. Mol Microbiol* **45**, 1177–1190

Voisard, C., Keel. C., Haas D., Defago, G. (1989) Cyanide production by *Pseudomonas fluorescens* helps auppress black root-rot of tobacco under gnotobiotic conditions. *EMBO J* **8**: 351–358.

Walker, C., Goodyear, C., Anderson, D., Titball, R.W. (2000). Identification of arsenic- resistant bacteria in the soil of a former munitions factory at Locknitz, Germany. *Land Contamination Reclamation*, **8**, 13–18.

Wandersman, C. and Delepelaire, P. (2004). Bacterial iron sources: from siderophores to emophores. *Annu Rev Microbiol* **58**, 611–647.

Wang, N., Lu, S. E., Records, A. R., Gross, D. C. (2006). Characterization of the transcriptional activators SalA and SyrF, which are required for syringomycin and syringopeptin production by *Pseudomonas syringae* pv. *syringae*. *J. Bacteriol*. **188,** 3290-3298.

Wei, H.L. and Zhang, L.Q. (2006). Quorum-sensing system infuences root colonization and biological control ability in *Pseudomonas fluorescens* 2P24 *Antonie van Leeuwenhoek* **89**: 267–280.

Whitehead, N.A., Barnard, A.M., Slater, H., Simpson, N.J., Salmond, G.P. (2001). Quorum-sensing in Gram-negative bacteria. *FEMS Microbiol Rev* **25**: 365–404.

Wiebe, W. L., and R. N. Campbell. 1993. Characterization of *Pseudomonas syringae* pv. *maculicola* and comparison with *P.s. tomato*. Plant Dis. **77:**414– 419.

Willis, D. K., and Kinscherf, T. G. (2004). Global regulation in *Pseudomonas syringae*. *Pseudomonas* Vol. 2, Virulence and Gene Regulation. J.-L. Ramos, ed. Kluwer Academic/Plenum Publishers, New York, U.S.A. pp 223-238.

Winsor GL, Van Rossum T, Lo R, Khaira B, Whiteside MD, Hancock REW & Brinkman FSL (2009). *Pseudomonas* Genome Database: facilitating user-friendly, comprehensive comparisons of microbial genomes. *Nucleic Acids Res* **37**: D483–D488.

Wissing, F., and K. S. Andersen. (1981). The enzymology of cyanide production from glycine by a *Pseudomonas* species. Solubilization of the enzyme, p. 275–287. *In* B. Vennesland, E. E. Conn, C. J. Knowles, J. Westley, and F. Wissing (ed.), Cyanide in biology. Academic Press Ltd., London, England

Withers, H., Swift, S., Williams, P. (2001). Quorum sensing as an integral component of gene regulatory networks in Gram negative bacteria. *Curr Opin Microbiol* 186–93.

Wong, W.C. and Preece, TF. (1979). Identification of *Pseudomonas tolaasii*: the white line in agar and mushroom tissue block rapid pitting tests. Appl. Bacteriol. **47**:401– 407.

Yamamoto, S., Kasai, H., Arnold, D.L., Jackson, R.W., Vivian, A., Harayama, S. (2000). Phylogeny of the genus *Pseudomonas*: Intrageneric structure reconstructed from the nucleotide sequences of *gyrB* and *rpoD* genes. Microbiology, **146**, 2385–2394.

Yang L, Harroun. T.A., Weiss, T.M., Ding L.,,Huang, H.W. (2001) Barrel-Stave Model or Toroidal Model? A Case Study on Melittin Pores. *Biophysical Journal* **81**: 1475–1485.

Yu, J., Peñaloza-Vazquez, A., Chakrabarty, A.M., Bender C.L. (1999). Involvement of the exopolysaccharide alginate in the virulence and epiphytic fitness of *Pseudomonas syringae* pv. *syringae*. *Molecular Microbiology*, **33**, 712-720.

Zhang, L., and J. Y. Takemoto. 1987. Effects of *Pseudomonas syringae* phytotoxin, syringomycin, on plasma membrane functions of *Rhodotorula pilimanae*. *Phytopathology* **77:**297–303.

Zhang, L.H., Murphy, P.J., Kerr, A., Tate, M.E. (1993). *Agrobacterium* conjugation and gene regulation by *N*-acyl-L-homoserine lactones. *Nature*. **362**:446-448.

Zhou, T. and Paulitz, T.C. (1993) *In vitro* and *in vivo* effects of *Pseudomonas* spp. on *Pythium aphanidermatum*: zoospore behavior in exudates and on the rhizoplane of bacteria-treated cucumber roots. *Phytopathology*, **83**, 872–876.

Zhou, T. and Paulitz, T.C. (1994). Induced resistance in the biological control of *Pythium aphanidermatum* by *Pseudomonas* spp. on European cucumber. *J. Phytopathol.* **142**, 51–63.

Zinn, M., Witholt, B., Egli, T. (2001). Occurrence, synthesis and medical application of bacterial polyhydroxyalkanoate. *Adv Drug Deliv Rev* **53**:5–21.