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Phenotypic and transcriptional analysis of secondary metabolites production in *Pseudomonas corrugata* CFBP5454

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SUMMARY

Natural products have received a great deal of attention as sustainable alternative for management of plant diseases. In this study the potential and actual arsenal of secondary antimicrobial metabolites of the bacterium *Pseudomonas corrugata,* an ubiquitous bacterium, was dissected by phenotypic assays, genome mining and transcriptional studies. *Pseudomonas corrugata* strain CFBP 5454 was shown to produce diffusible compounds that inhibit the growth of plant pathogenic fungi and bacteria and antifungal volatile compounds and to produce cyanide. Mutant derivatives in LuxR transcriptional regulators, i.e. *P. corrugata* GL2 (*pcoR* mutant) and GLRFIA (*rfiA* mutant) impaired in cyclic lipopeptide (CLP) production, showed a diffusible compound-mediated reduced activity, depending on the biocontrol strain, challenge microorganism and culture medium but clearly indicate the production of additional antimicrobial substances.

PcoR is the cognate receptor of the N-acyl-homoserine lactones, synthetised by PcoI in *P. corrugata* quorum sensing (QS) system. The QS system also directly activates the transcriptional regulator gene *rfiA***.** In order to define the complete regulon of PcoR and RfiA, deep sequencing of cDNA library (RNA-seq) was used to analyse the whole transcriptomes of GL2 (*pcoR*-) and GLRFIA (rfiA-) mutants at early stationary phase of growth on a minimal medium. Differential expression analysis between the Wt and the mutants showed that the CLP cormycin and corpeptins biosynthetic clusters as well as that of an additional putative bioactive peptide and the exopolisaccharide alginate are under the control of the PcoR-RfiA regulon. Quantitative Real-time PCR assays demonstrated that CLP genes are upregulated in minimal medium and plant growth conditions as compared to reach medium cultures, whereas alginate production and

biosynthesis gene expression were upregulated in rich growth conditions. In addition this study shows that citronellol is able to inhibit the growth of plant pathogenic bacteria but also could interfere with Quorum sensing system mediated by medium chain AHL in the biosensor strain *Chromobacterium violaceum* and in *P. corrugata* with QS system and the expression of genes contributing to the production of CLPs.

RIASSUNTO

I prodotti naturali sono oggetto di grande interesse come alternativa sostenibile per la gestione delle malattie delle piante. In questo studio è stato valutato, mediante saggi fenotipici, analisi del genoma e studi di trascrizione, il potenziale ed effettivo arsenale di metaboliti secondari antimicrobici prodotti dal batterio ubiquitario *Pseudomonas corrugata*. E' stato dimostrato che, il ceppo di *P. corrugata* CFBP 5454, produce composti diffusibili che inibiscono la crescita di funghi e batteri patogeni e composti volatili antifungini tra cui acido cianidrico. Ceppi batterici inattivati nei geni che codificano per i regolatori trascrizionali LuxR, *P. corrugata* GL2 (*pcoR*-) e GLRFIA (*rfiA*-), in cui la produzione di lipopeptidi ciclici (CLP) risulta alterata, hanno mostrato un'attività antimicrobica ridotta, a seconda del ceppo batterico, patogeno e substrato, ma indicano chiaramente la produzione di ulteriori sostanze antimicrobiche. PcoR è il recettore degli acil-omoserina lattoni sintetizzati da PcoI nel sistema di regolazione Quorum sensing (QS). Il sistema QS, inoltre, attiva direttamente il gene *rfiA* codificante per un altro regolatore trascrizionale della famiglia LuxR. Al fine di definire quali siano i geni regolati da PcoR ed RfiA, è stato analizzato il trascrittoma dei mutanti GL2 (*pcoR*-) e GLRFIA (*rfiA*-)

attraverso il sequenziamento delle librerie di cDNA, ottenute da colture all'inizio della fase stazionaria coltivate in substrato minimo. L'analisi dei geni differenzialmente espressi tra il ceppo parentale e i mutanti, ha dimostrato che i cluster biosintetici dei CLP cormicina e corpeptine, e quello di un'addizionale putativo peptide bioattivo, nonchè dell'esopolisaccaride alginato, sono sotto il controllo trascrizionale del regulone PcoR-RfiA. Saggi di Real-time PCR quantitativa hanno dimostrato che i geni CLP sono sovraespressi in condizioni di crescita su substrato minimo e in pianta rispetto a colture in substrato ricco, mentre i geni responsabili della biosintesi di alginato sono sovraespressi in condizioni di crescita su substrato ricco. Inoltre, in questo studio, è stato dimostrato che il citronellolo è in grado di inibire la crescita dei batteri fitopatogeni e i risultati suggeriscono possa interferire con il sistema di regolazione Quorum sensing mediato da AHL a catena media del ceppo biosensore *Chromobacterium violaceum,* e con quello di *P. corrugata* interferendo indirettamente con i geni che contribuiscono alla produzione di CLP in *P. corrugata*.

1. INTRODUCTION

1.1. Plant associated *Pseudomonas*

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). 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 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.

Phytopathogenic *Pseudomonas* are a very diverse group of bacteria with respect to their genetics, ecology, and the kinds of diseases they cause. They currently are grouped into approximately numerous species. Moreover, there are

approximately 50 pathovars in the species *P. syringae*, most of which attack different hosts. There also are pathovars in other species. These pseudomonads are worldwide in distribution and cause diseases on most major groups of higher plants. Some of the world's most serious bacterial diseases are caused by pseudomonads. Because of the genetic diversity of the phytopathogenic pseudomonads, disease symptomatology in plants ranges from necrotic lesions, spots, cankers, and twig dieback (blights) to hyperplasias (galls, scabs), tissue maceration (rots), and vascular infections (wilts). Thus they are diverse in the symptoms they induce as well as in their invasion strategies and mechanisms of pathogenesis (Hofte and De Vos, 2006).

1.2. *Pseudomonas corrugata*

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). *P. corrugata* is considered to be a weak and opportunistic pathogen. Nevertheless, infections involving up to 100% of the plants have been observed and leading to loss of crops or severe economic losses. In addition, the infections have been related to particular growing conditions (CABI, 2006).

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).

1.2.1. Taxonomy

P. corrugata belong to the Gammaproteobacteria (Kersters *et al.,* 1996), and to the genus *Pseudomonas sensu stricto* which includes species within rRNA similarity group I or the *fluorescens* rRNA branch (De Vos *et al.,* 1985). Phylogenetic analysis based on 16S rRNA gene sequences placed *P. corrugata* within the *P. fluorescens* branch (Moore *et al*., 1996; Anzai *et al*., 2000). Other studies using the combined nucleotide sequences of the *rpoD* and *gyrB* genes included it 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 further studies based on a MLSA approach using 16S rRNA gene, *rpoB*, *rpoD* and *gyrB* gene sequences both with a few species and a collection of 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* (Cladera *et al.,* 2007; Mulet *et al.,* 2010), with *P. mediterranea* as the most related species to *P. corrugata.*

More recently Trantas *et al.,* (2015 b) applied the MLST approach with sequences spanning the coding regions of *fruK*, *gltA*, *gyrB*, *mutL*, *rpoB*, and *rpoD* using several other strains belonging to different phylogenetic clades. The phylogenetic analysis showed that the *Pcor* and *Pmed* strains cluster in distinct monophyletic clades within the larger *P. fluorescens* clade, clearly differentiated from *P. brassicacearum* subsp. *brassicacearum* NFM421T and *P. fluorescens* F113 and in the *P. fluorescens* lineage along with *P.mediterranea*, *P.*

kilonensis, *P. thivervalensis,* and *P. brassicacearum* (the so called *P. corrugata* subgroup).
Similar results were generated by Trantas *et al.* (2015 a) using the whole genome phylogenetic tree from four *P. corrugata* strains and the closely related *P. mediterranea* (5 strains) and *P. brassicacearum* subsp. *brassicacearum* NFM421, *P. fluorescens* A506, *P. fluorescens* CHA0, *P. fluorescens* F113, *P. fluorescens* Pf0-1, *P. fluorescens* Pf-5, *P. fluorescens* SBW25, and *P. syringae* pv. *syringae* B728a and *P. syringae* pv. *tomato* DC3000 as out-groups (Fig. 1).
It is notheworty that *Pseudomonas* species or strains closely related to *P. corrugata* whose taxonomic position need sometimes to be clarified, were isolated from the rhizosphere or from agricultural soils and mostly studied as bio-control strains of soil-borne plant diseases. Notably, *P. corrugata* and *P. mediterranea* are the only non-fluorescent and the only plant pathogenic species placed in this group, to date. *P. brassicacearum* has been described as a major root-associated bacterium of *Arabidopsis thaliana* and *Brassica napus,* which may co-exist with *Pcor* and *Pmed* in agricultural niches (Ortet *et al.,* 2011). *P. fluorescens* F113 is a plant growth-promoting rhizobacterium that has biocontrol activity against fungal plant pathogens; it was recently transferred to the *P. brassicacearum* group (Redondo-Nieto *et al.,* 2013).

1.2.3. Species description

P. corrugata is a Gram-negative, non spore forming rods. Is strictly aerobic, do not produce fluorescent pigments, and is oxidase positive, reduce nitrates, do not produce levan and it isn't 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). 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). This specie 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). 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).

1.2.4. The closely related species *P. mediterranea*

A polyphasic approach revealed that two closely related but distinct taxa were present within the *P. corrugata* species. The taxon which contained the type strain CFBP2431T maintained the epithet *P.corrugata*, while the other strains included were assigned to a novel species, named *P. mediterranea* (Catara *et al.,* 2002). The two species are phenotypically distinguishable by the ability of *P. corrugata*, but not of *P. mediterranea*, to utilize histamine 2-ketogluconate and meso-tartrate as a unique carbon source. The two species can also be clearly distinguished by 16S rDNA analysis, by means of DNA-based fingerprinting methods (Catara *et al.,* 2000, 2002) and by multi-locus sequence analysis (MLSA) (Trantas *et al.,* 2015 a). Multiplex, conventional, or real-time PCR can also be used to screen tomato planting materials for detection of, and discrimination between, the two bacterial species (Catara *et al.,* 2000; Licciardello *et al.,* 2011).

1.2.5. Draft genome sequence

The first draft genome sequence was obtained for strain CFBP5454 by Licciardello *et al.* (2014). The genomic sequences of other 3 *P. corrugata* strains were obtained in a

comparative genomic study together with those of 5 *P.mediterranea* strains (Trantas *et al.,* 2015 a). The four strains contain 84–442 contigs with an N75 ranging from 16,551 to 85,837. The total number of nucleotides assembled in contigs ranged for *P. corrugata* from 6,084,011 to 6,266,776. GC content ranging from 60.1% to 60.6%. Other characteristics were shown in Table 1 extrapolated from the *P. corrugata* and *P. mediterranea* comparative study (Trantas *et al.,* 2015 a).

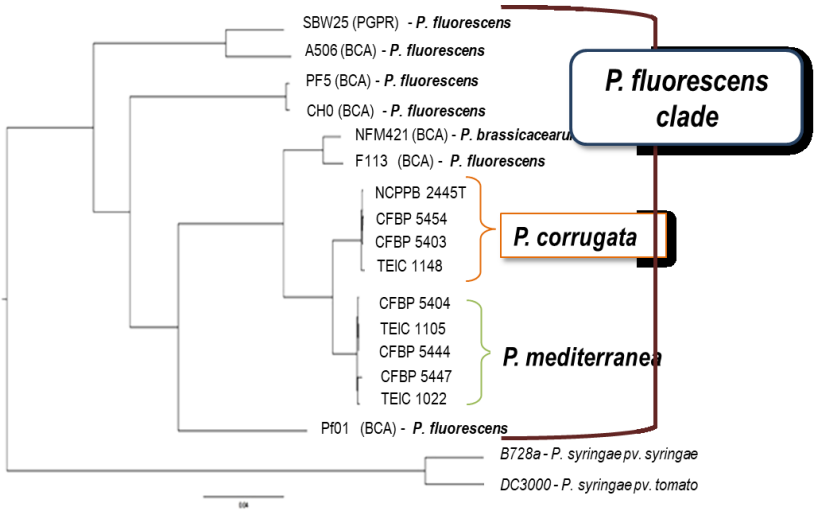

Figure 1. Phylogenetic tree of *Pseudomonas* species. All nodes had 100% bootstrap support, except the one separating *Pmed* CFBP5447 and *Pmed* TEIC1022, with 98% bootstrap support. The tree was generated using whole genome alignment of orthologous proteins following using FigTree (http://tree.bio.ed.ac.uk/software/figtree/) (Trantas *et al.,* 2015 a).

Table 1. Genomic statistics for *Pseudomonas corrugata* strains in Trantas *et al.,* 2015 a.

	CFBP5403	**CFBP5454**	**NCPPB2445^T**	**TEIC1148**
Total number of reads	11,800,202	8,420,692	1,519,930	8,608,470
Average read length	35	70.6	145	35
Total bases	413,007,070	594,525,036	220,389,850	301,296,450
DNA, total n.of bases in contigs	6,166,582	6,195,615	6,084,011	6,266,776
Ambiguous bases	39,282	2299	3749	31,312
% of ambiguous bases	0.64	0.04	0.06	0.50
DNA coding number of bases	5,471,555	5,491,990	5,391,923	5,567,198
DNA G+C number of bases	3,706,298	3,747,457	3,686,460	3,763,425
GC content (%)	60.1	60.5	60.6	60.1
Total number of contigs	442	84	104	432
N75	17,365	48,599	85,837	16,551
N50	29,882	79,475	202,143	32,179
Genes total number	5912	5570	5463	5845
Coding density (%)	87.9	88.5	88.4	88.4
Completeness (%)	97.02	99.69	99.68	99.14
Contamination (%)	0.80	0.42	0.45	0.47
Protein coding genes	5809	5443	5313	5737
RNA genes	103	127	150	108
Protein coding genes with function prediction	4812	4621	4516	4753
Protein coding genes without function prediction	997	822	797	984
Protein coding genes for enzymes	1307	1355	1341	1331
w/o enzymes but with candidate KO based enzymes	147	19	26	68
Protein coding genes connected to transporter classification	832	792	777	810
Protein coding genes connected to KEGG pathways	1497	1564	1546	1536
Protein coding genes connected to KEGG Orthology (KO)	2733	2850	2807	2808
Protein coding genes connected to MetaCyc pathways	1272	1325	1311	1300
Protein coding genes with COGs	3839	4012	3927	3923
Chromosomal cassettes	717	532	508	697
Biosynthetic clusters	39	46	38	45
Genes in biosynthetic clusters	289	386	332	331
COG clusters	2033	2051	2046	2053
KOG clusters	841	841	852	852
Pfam clusters	2601	2566	2551	2597
TIGRfam clusters	1345	1362	1355	1369

TEIC, Technological Educational Institute of Crete Bacterial collection; CFBP, International Center for Microbial Resources, French Collection for Plant-associated Bacteria, INRA, Angers, France; NCPPB, National Collection of Plant Pathogenic Bacteria, Fera, York, U.K.

1.3. *P. corrugata* and Tomato pith necrosis

1.3.1. Symptoms and biology

Catara (2007) collected available information on the disease in a review and from than no important achievement were obtained on the study of the disease. TPN is the characteristic symptom of a tomato disease where the 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. 2).

Figure 2. Typical symptoms of Tomato Pith Necrosis induced by *P. corrugata* spp. The pith appears 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

Usually, the first visible symptom is chlorosis and withering of the youngest leaves, followed by loss of turgor and eventual collapse of the whole plant in later stages of the disease. 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.

Infections are more evident when the first trusses ripen on the tomato plants. Initially, only slight wilting in the hottest part of the day and at times chlorosis of the apex can be seen. Hydropic or dark areas may be present on the epidermis and may extend along the entire stem. Occasionally, traces of bacterial ooze can be observed on the leaf scar. Moreover, the stem presents long conspicuous adventitious roots even 1–2 m above the soil, and these may allow differential diagnosis.

Cultivated varieties of *Lycopersicon esculentum* are more susceptible to attack than wild *Lycopersicon* spp. (Scortichini and Rossi, 1993). Symptoms on pepper and chrysanthemum are similar to those observed on tomato. Injections of a bacterial suspension of isolates of *P. corrugata* have caused pith necrosis in aubergine, bean, celery, cucumber, melon, pea, tobacco and courgette (not all the strains) (Catara *et al.,* 1997, 2002; Sutra *et al.,* 1997). The disease occurs world-wide in all tomato-growing areas and can cause severe crop losses. Tomato plants artificially inoculated with a *P. corrugata* strains showed reduced total tomato yield and fruit size (Moura *et al.,* 2005).

P. corrugata is able to survive in soil and in the rhizosphere of the host plant species and of other plant species that can represent reservoirs for new infections. *P. corrugata* survived longer in sandy-loam soil than in sandy soil. In bulk soil the bacterial populations declined within 10 weeks

as compared with soil in which tomato plants were transplanted where the bacterial population density remained constant (Bella *et al.,* 2003). Nevertheless, in bulk soil *P. corrugata* was still detected after enrichment in liquid media. The bacterium is able to colonize tomato rhizosphere and to start endophytic colonization starting from the inoculum present in soil as well as from inoculated seeds (Bella *et al.,* 2003; Cirvilleri *et al.,* 2000).

P. corrugata enters the host through wounds on the stem, collar and roots (Bella *et al.,* 2003; Nauman *et al.,* 1989; Scarlett *et al.,* 1978; Scortichini, 1989). Scanning electron microscopy (SEM) showed that *P. corrugata* cells colonizing parenchymal tissues of tomato plants are consistently associated presumably with exopolysaccharide that embedded them to the cell wall surface (Coco *et al.,* 2001). *P. corrugata* has been reported to produce alginate (Fett *et al.,* 1996). The advancing border of infection is from 3 to 6 cm inward from the margin of visible symptoms with bacterial population concentrations of about 10^5 CFU/g in symptomless tissue and of two and three logs higher in symptomatic tissue (Cirvilleri *et al.,* 2000). Stem colonization could also proceed towards the xylem and the epidermis where the bacterium exits in the form of bacterial ooze under conditions of high humidity.

Some evidence suggests that the bacterium may be seed-borne. The bacterium from infected plants can be dispersed by rain splash, sprinkler splash and dew, or during handling. It can efflux from the roots into the water of the circulating solution of soilless system and hence infect healthy plants (Fiori, 2002; Naumann *et al.,* 1989; Sadowska-Rybak *et al.,* 1997). Irrigation water may play a role in the dissemination of the pathogen (Scarlett *et al.,* 1978).The disease has been observed in the open field and under cover but the

conditions in the latter seem to favour the disease. The presence of free water on leaves and stem surfaces and high soil nitrogen levels, which lead to more tender vegetation, promote *P. corrugata* infection (Carroll *et al.,* 1992; Naumann *et al.,* 1989; Scarlett *et al.,* 1978).

1.3.2. Bacterial isolation and molecular diagnosis

P. corrugata is easily isolated from infected plants on bacteriological agar media (YPGA, NDA). Accurate identification of *P. corrugata* is important because other bacteria (mainly some *Pseudomonas* spp. but also *Pectobacterium* spp.) have also been associated with necrosis of tomato, even if secondary symptoms (exudates, adventitious roots) have not been reported in such cases. Biochemical tests can be performed to provide bacterial identification and these can be further supported by pathogenicity assays on tomato plants and to some extent by hypersensitivity reaction (HR) on tobacco (Catara *et al.,* 2002; Sutra *et al.,* 1997).

Due to the intraspecific variability observed, detection or identification using serology is not advisable. Two pairs of specific primers were designed from the sequences of two RAPD-PCR-generated fragments each being specific either for *P. corrugata* or for *P. mediterranea* either when used as probes or when amplified by PCR with primers designed at the two extremities (Catara *et al.,* 2000). No cross-reaction was observed against other *Pseudomonas* spp. or tomato bacterial pathogens. *P. corrugata* and *P. mediterranea* could be individually identified starting from either bacterial DNA or whole cells, and detected in plants by mul-tiplex PCR assay that amplifies one of the two possible amplicons (Catara *et al.,* 2000; Catara *et al.,* 2002). Sequences internal to the same target DNAs were used to

develop a duplex real-time PCR assay able to detect and quantify both species in planta by monitoring the fluorescence of two specific TaqMan probes labelled with different fluorochromes (Licciardello *et al.,* 2007).

1.4. *P. corrugata* as biocontrol agent (BCA)

A number of *P. corrugata* strains were isolated from the root environment and were investigated as rhizosphere competent biocontrol agents. *In vitro* antagonistic 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).
Some *P. corrugata* strains have been successfully tested as a biological control agent in different pathosystems (Table 2). *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).

Guo *et al.,* 2007 isolated a *Pseudomonas* strain designated P94 from agricultural soil samples in Beijing. This strain exhibited an important antagonistic activity against *Botrytis cinera* and other phytopathogenic fungi (*C. fimbriata*, *M. laxa*, *M. grisea*, *P. aphanidermatum*, *P. capsici*, *A. solani*, *R. cerealis*, *F. oxysporum* f. sp. *cucumerinum*, *F. oxysporum* f. sp. *vasinfectum*, *F. oxysporum* f. sp. *lilii*) and bacteria (*P. corrugata* ICMP 5819, *P. syringae*, *A. avenae*, *R. solanacearum*).

P94 showed inhibition activity to tomato grey mildew by tomato leaf testing in vitro. Strain P94 showed a positive reaction for HCN, protease, and phosphatase production and indole acetic acid tests and a negative reaction for siderophore-, chitinase-, and cellulase-production tests. Sang *et al.* (2008) demonstrated that *P. corrugata* strains CCR04 and CCR80 are suppressive to *Phytophthora* blight of pepper caused by *P. capsici*. Later they showed that this *P. corrugata* strains effectively reduced the severity of disease caused by *P. capsici* through bacterial colonization on pepper roots. The results revealed that root colonization by these strains might be promoted by increased biofilm formation, enhanced motility ability (swimming and swarming activities) as well as reduced sensitivity to oxidative species such as H_2O_2 (Sang and Kim, 2014). This

phenomenon was reported in previous studies, that is, increased microbial activity was associated with suppression of plant diseases, such as damping-off in cucumber caused by *P. ultimum* and *Phytophthora* blight of pepper caused by *P. capsici* (Chen *et al.,* 1988; Kim *et al.,* 2012; Sang and Kim, 2012).

Mendes *et al.* (2011) isolated *Pseudomonas* sp. SH-C52 from the rhizosphere of sugar beet plants grown in a soil suppressive to the fungal pathogen Rhizoctonia solani and showed that its antifungal activity is, in part, attributed to the production of the chlorinated 9-amino-acid lipopeptide. Phylogenetic analysis placed strain SH-C52 within the *Pseudomonas corrugata* clade. To get more insight into its biosynthetic repertoire, Van Der Voort *et al.* (2015) sequenced the genome of *Pseudomonas* sp. SH-C52 and subjected to in silico, mutational and functional analyses. In silico analysis for secondary metabolites revealed a total of six non-ribosomal peptide synthetase (NRPS) gene clusters, including the two previously described NRPS clusters for thanamycin and the 2-amino acid antibacterial lipopeptide brabantamide. The authors showed that thanamycin also has activity against an array of other fungi (*R. solani*, *B. cinerea*, *Geotrichum* sp., *P. infestans*), *Bacillus megaterium* and the Gram-negative bacteria *Pseudomonas syringae* and *Pectobacterium atrosepticum.* Brabantamide A exhibited anti-oomycete activity (*Phytophthora capsici*, *Pythium ultimum*) and affects phospholipases of the late blight pathogen *Phytophthora infestans*. Most notably, mass spectrometry led to the discovery of a third lipopeptide, designated thanapeptin, with a 22-amino-acid peptide moiety. Seven structural variants of thanapeptin were found with varying degrees of activity against *P. infestans*. 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).

Table 2. *Pseudomonas corrugata* as a biocontrol agent: *in vivo* experiments under laboratory or field conditions (modified from Catara, 2007).

Plant species	Disease	Pathogen	Strain	Experiment information	References
Cucumber	*Pythium* root rot	*Pythium aphanidermatum*	13	Growth bench/pot; Greenhouse/soilless culture	Zhou and Paulitz (1994), McCullagh *et al.* (1996)
Cucumber	Damping off	*Pythium ultimum*	R 117	Growth chamber and greenhouse/pot	Georgakoupoulus *et al.* (2002)
Grapevine	Grey mould	*Botrytis cinerea*	Various	Post-harvest/detached berrei	Cirvilleri *et al.* (2001)
Grapevine	Crown gall	*Agrobacterium tumefaciens*	Various	Field	Bell *et al.* (1995)
Lemon	Green mould	*Penicillium digitatum*	Various	Postharvest	Smilanick and Denis Arrue (1992), Cirvilleri *et al.* (2001)
Lemon	Malecco	*Phoma tracheiphila*	Various	Greenhouse/pot	V. Catara (unpublished)
Maize	Damping off	*Pythium ultimum*	NRRL B-30409	Pot	Pandey *et al.* (2001)
Maize	PGPR	—	NRRL B-30409	Greenhouse/trays	Trivedi *et al.* (2005)
Nectarine, peaches	Brown rot	*Monilia fructicola*	ATCC 29736	Post-harvest	Smilanick *et al.* (1993)
Pea	Root diseases	*Rhizoctonia* spp.; *Aphanomyces*	Various	Field	Chun *et al.* (1998)
Pepper	*Phytophthora* blight	*Phytophthora capsici*	CCR04-CCR80	Growt chamber and Field	Sang *et al.* (2008)
Potato	Silver scurf	*Helminthosporium solani*	Various	Tubers and field potato	Chun and Shetty (1994)
Potato	Ring rot	*Clavibacter michiganensis* ssp. *sepedonicus*	Various	Tubers and field potato	Schroeder and Chun (1995)

Plant species	Disease	Pathogen	Strain	Experiment information	References
Potato	*Fusarium* dry rot	*Giberella pulicaris*	Various	Tubers	Schilsler *et al.* (1997)
Potato	Wilt	*Verticillium dahliae*	—	Pot	Entry *et al.* (2000)
Sugar beet	*Pythium* root rot	*Pythium ultimum*	2140luxAB	Growth chamber/ microcosm	Schmidt *et al.* (1997, 2004)
Sugar beet	Damping off	*Pythium ultimum*	R 117	Growth chamber/pot	Georgakoupoulus *et al.* (2002)
Sugar beet	*Rhizoctonia root*	*Rhizoctonia solani*	SH-C52	growth chamber/pot	Mendes *et al.* (2011)
Tomato	*Fusarium* wilt	*Fusarium oxysporum* f.sp. *lycopersici*	Various	Pot	Larkin and Fravel (1998)
Tomato	Grey mildew	*Botrytis cinerea*	P94	Leaf testing in vitro	Guo *et al.* (2007)
Wheat	Take-all	*Gaemannomyces graminis* var. *tritici*	2140	Field	Ryder and Rovira (1993)
Wheat	Take-all	*Gaeumannomyces graminis* var. *tritici*	2140R	Greenhouse/pot	Barnett *et al.* (1998), Ryder *et al.* (1999)
Wheat	*Rhizoctonia* root rot	*Rhizoctonia solani*	2140R	Greenhouse/pot	Ryder *et al.* (1999)
Wheat	Take-all	*Gaeumannomyces graminis* var. *tritici*	2140RlacZY	Field	Choi *et al.* (2003)
Wheat	*Pythium* root rot	*Pythium* spp.	—	Pot	Chun and Gao (1995)

1.5. *P. corrugata* and Poly(hydroxyalkanoates) 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). 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).

P. corrugata PHA composition was first investigated on strain 388 isolated an 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). 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 and soy molassa (Solaiman *et al.,* 2006).

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. Conte *et al.,* (2006) examined the polyhydroxyalkanoate (PHA) synthases *phaC1* and *phaC2* gene expression in two strains (388 and CFBP 5454) 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. 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.

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).

Recent studies have been concentrated in order to exploit the use of crude and pure glycerol as carbon sources for PHA production in P. *corrugata* CFBP5454 and the closely related bacteria *P. mediterranea* CFBP5447. The best PHA/dried cells ratios have been obtained from *P. mediterranea* when crude glycerol was supplied at 2 %, giving more than 60 % PHA, higher than that produced by *P. corrugata* CFBP5454 which reached 47.3 % from crude glycerol and 27.9 % from refined glycerol (Palmeri *et al.,* 2012). Diversely, both strains of *P. corrugata* produce an elastomer mcl-PHA very similar regardless of carbon source whereas *P. mediterranea* a distinctive and transparent filmable polymer with low molecular weight (56,000 Da) from refined glycerol showing enhanced properties that look promising for an improvement of the technological properties required for application as paper and tissue coatings. In particular these properties suggests a potential application as additive for blending with other biopolymers, expecially with PLA (Pappalardo *et al.,* 2014, Botta *et al.,* 2015).

According to preliminary GC/MS profiles, the composition of the mcl-PHAs produced using glycerol are quite different from the one previously obtained by *P. corrugata* CFBP5454 conversion of waste fried cooking oils as carbon source (Alicata *et al.,* 2005). They showed different monomeric units of side chains, which range from C12 to C19 in length in mcl-PHA(CG), and from C5 to C16 in mcl-

PHA(RG). Since also the conversion efficiency of *P. mediterranea* was different from *P. corrugata* strains the results confirm that the two species have a different metabolic pathway as suggested by Solaiman *et al.* (2005, 2007), according to granules accumulation inside the cells growing on oleic acid.

These differences have been confirmed also by transcriptional studies by which profiles of *P. corrugata* CFBP 5454 and *P. mediterranea* CFBP 5447 in the early stationary phase of growth, using high-grade glycerol as the sole carbon source and limiting the inorganic nutrients, have been compared. RNA-seq analysis showed they undergo a different modulation of genes belonging to diverse functional categories involved in central metabolic pathways related to PHA biosynthesis which contributes to understanding some aspects of the differences in composition and physical properties observed in mcl-PHAs, although not conclusive (Licciardello *et al.,* 2016). Particularly interesting was the up-regulation of genes involved in alginate biosynthesis in *P. mediterranea* in comparison to P. *corrugata* confirmed by the large amount of EPS produced (Licciardello *et al.,* 2016).

1.6. First studies on *P. corrugata* interaction with plant and microrganisms

1.6.1. Hypersensitivity response

Studies performed more than twenty years ago pointed out that 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). The authors also established that elicitation of HR by HR2 cannot be ascribed to its functioning as a toxin because it did not cause TPN.
Later it has been demonstrated that *P. corrugata* produces in culture phytotoxic and antimicrobial cationic cyclic lipopeptide (CLPs): corpeptins A and B and cormycin A that will be thoroughly described below (Emanuele *et al.,* 1998; Scaloni *et al.,* 2004).

1.6.2. Secondary metabolites

P. corrugata's efficacy as a biological control agent seemed linked to its elevated rhizosphere competence. In addition, its *in vitro* antimicrobial activity against a long list of microorganisms 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 of 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).

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

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

Corpeptins (CPs) were isolated from the culture filtrates of *P. corrugata* type strain NCPPB 2445 isolated from tomato (Emanuele *et al.,* 1997). 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 primary structure of the peptide moiety was: Dhb Pro Ala Ala Ala VaI 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.

In the tobacco leaf assay CPs induced chlorosis 72 h after tissue infiltration with CP A more phyotoxic than CP B. CPs

showed inhibitory activity against *Bacillus megaterium* and not to *Rhodotorula pilimanae.*

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.

Cormycin A (CM A) was isolated from the culture filtrate of *P. corrugata* strain IPVCT 10.3. 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.

1.6.4. Alginate production

The ability to produce a variety of acidic exopolysaccarides (EPSs) including alginate (a polyuronan) as well as the neutral polymer levan (a fructan) have been demonstrated for the group I of pseudomonads (Gross and Rudolph, 1987; Russel and Gacesa, 1988; Fett *et al.,* 1995). The term alginate encompasses a group of structurally-related linear, acidic polysaccharides containing varying amount of β-1,4-linked D mannuronic acid and its C-5 epimer α-L-glucuronic acid. Bacterial alginates have been studied as possible substitute for algal alginates for certain commercial applications. In a previous study, Fett *et al.,* 1986, were able to induce EPS production in *P. corrugata* NCPPB 2445. Southern blot hybridization studies demonstrated that *P. corrugata* 388 contained genes with homology to alginates biosynthetic and regulatory genes cloned from the alginates-producing bacteria *P. aeruginosa* (Fett *et al.,* 1992).

Screening studies onEPS production showed that three *P. corrugata* strains (388, 717, ATCC 29736^{T}) produced EPSs after growth on PAF with 5% glycerol and showed a mucoid appearance after 4-5 days of incubation. Yield of EPS per culture dish after isopropanol precipitation was 2-4 mg. Colorimetric analyses of partially purified EPSs isolated from the three strains indicated that they all contained high levels (greater than 40%, w/w) of uronic acid and no significant amount of sugars were detected. Further purification analysis of EPS produced by *P. corrugata* 388 revealed the presence of solely uronic acid (100% w/v) and after reduction and GLC analysis the presence of mannose alone has been detected (Fett *et al.,* 1996). This indicated that P. *corrugata* 388 produced alginate as polymannuronic acid and was 10% acetylated. These traits are common with

other alginates produced by pseudomonads (Russel and Gacesa, 1988).

1.7. *Pseudomonas corrugata* AHL Quorum Sensing system

1.7.3. The PcoI/R AHL-QS system

P. corrugata strains were first reported as potentially being able to produce N-acylhomoserinelactones (AHLs) as inter cellular signal molecules during studies dealing on screening of strains of different *Pseudomonas* species (Dumenyo *et al.,* 1998; Elasri *et al.,* 2001). Licciardello *et al.,* (2007) demonstrated, for the first time, testing a representative collection of strains that *P. corrugata* possess a very well conserved AHL-QS system involved in the regulation of microbiological and phytopathogenic traits of this bacterium. The authors demonstrated that despite their heterogeneous phenotypic and genotypic characteristics, all the strains produce comparable levels of the same AHLs: C6 AHL, C6 3 oxo AHL and C8 AHL, with C6 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 of the genetic determinants encoding for an AHL-QS system designated *pcoI* and *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*. 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.

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 (2007) 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) (Licciardello *et al.,* 2007).

1.7.2 The transcriptional regulator RfiA

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. The latter is a well known biocontrol strain and the acronym of Regulator of fungal inhibition was retained also for CFBP5454 strain.

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) had a polar effect on the cotranscribed *rfiA* gene.

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 (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. 3). 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.
Analysis of *P. corrugata* genomes revealed that all strains have genes of only one canonical paired LuxI/R system (Trantas *et al.,* 2015 a). The QS gene topology was conserved in *Pcor* strains. All strains have the *rfiA* gene coding for a transcriptional regulator downstream of, and in the same orientation as, *luxI*. The LuxI and LuxR protein sequences are highly conserved in *Pcor* (between 99 and 100%).

1.7.3. Genes regulated by QS-RfiA

PcoABC transporter system

Downstream of *rfiA* three ORFs designated *pcoA, pcoB* and *pcoC, pcoABC* constitute an operon that encodes three components of a tripartite resistance nodulation cell division

(RND) transporter system (Licciardello *et al.,* 2009). This RND transporter system consist of an outer membrane protein (PcoA), a periplasmic membrane fusion protein (PcoB), and a cytoplasmic RND transporter (PcoC). The predicted PcoABC showed high homology to the PseABC efflux system described in *P. syringae* pv. *syringae* B301D, which is localized in the *syrsyp* 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 *pco ABC* transport system was not pivotal for virulence because GLPCOA was as virulent as the WT strain. 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. Licciardello *et al.,* (2009) 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* operone it her directly or indirectly (Licciardello *et al.,* 2009).

The operon CrpCDE

Part of the biosynthetic cluster responsible for corpeptin production, designated as *crpCDE* including genes transcriptionally joined coding for an NRPS and an ABC efflux system was described by Strano *et al.* (2015).These genes, and hence corpeptins, greatly contributed to *P. corrugata* virulence and plant interaction. The introduction of a mutation in *crpC* yielded a *P. corrugata* strain, PCONRPS, which failed to produce corpeptins, thus demonstrating that *crpC* is part of the corpeptin biosynthesis locus via a thiotemplate mechanism. Further analysis of the region downstream of *crpC* resulted in the identification of two other genes, which putatively code for an ABC transporter system. Gene disruption of *crpD* also affected the presence of corpeptins in the culture filtrates of *P. corrugata* CFBP 5454, supporting the assumption that crpDE is the unique transport system involved in corpeptin export. Phylogenetic analysis showed that CrpE groups with ABC transporters of bacteria that produce long-chain peptides. These transporters may have evolved differently from those involved in the secretion of CLP with shorter peptide chains or with linear peptides.

In terms of biological activity of corpeptins, Strano *et al.* (2015) found that the PCONRPS and PCOMFP mutant strains still release substances into the culture medium whose antimicrobial activity can be attributed to the

production of cormycin. Pathogenicity tests on tomato demonstrated that the mutant strains producing only cormycin were also clearly less virulent than the parent strain CFBP 5454, thus demonstrating the importance of corpeptins in the development of disease symptoms.
The results of leaf inoculations of *N. benthamiana* deserve separate consideration. When strain CFBP 5454 is infiltrated into *N. benthamiana* mesophyll, it induces the collapse and necrosis of the leaf tissue and, as in an HR, its population declines rapidly.
When tested for HR on *Nicotiana benthamiana* the mutant strains that did not produce corpeptins only caused chlorosis, and the population titre of the crpCDE mutant (PCONRPS strains) was invariable over a 4-day monitoring period. WT strain CFBP 5454 induces the collapse and necrosis of the leaf tissue and, as in an HR, its population declines rapidly. This result, and the fact that no T3SS was found in the *P. corrugata* CFBP 5454 genome (Licciardello *et al.* 2014), suggested that corpeptins play a role in the elicitation of HR in N. benthamiana. This is in accordance with recent studies, which showed that CLP may induce systemic resistance and which, taken together, suggest that CLPs constitute a novel class of microbial-associated molecular patterns (MAMPs) (reviewed in Raaijmakers *et al.,* 2010).
On the regulation point of view Strano *et al.,* (2015) also showed that the transcriptional regulators PcoR and RfiA play a pivotal role in the expression of crpC and crpD genes. QS is probably involved in the production of corpeptins, given the evidence that, in *P. corrugata* strain CFBP 5454, crpC transcription greatly increases with a high population density and following the trend in AHL signal molecule production. It presumably reaches large amounts of

transcripts as a consequence of the QS positive-feedback regulatory loop.

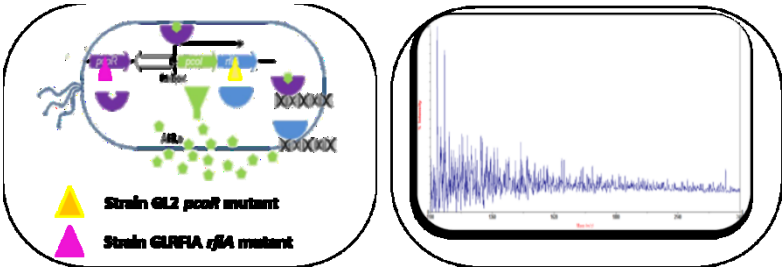

Figure 3. Working model for acyl-homoserine lactone (AHL) quorum-sensing (QS) and RfiA in *Pseudomonas corrugata* (A). QS allows bacterial populations to coordinate the expression of some traits in a cell-density-dependent manner. At high density of the population the signal molecules (AHL) produced by PcoI binds the cognate PcoR sensor/regulator, which activates or represses target gene expression. *rfiA* is cotranscribed with *pcoI* and encodes a transcriptional regulator of the LuxR family. GL2 and GLRFIA mutant strains have been inactivated in *pcoR* and *rfiA* genes, respectively. These genes encode for two LuxR transcriptional regulators, involved in CLPs production. No corpeptines and cormycin are present in the culture filtrates of GL2 and GLRFIA mutants, as revealed by MALDI-TOF analysis (B) (Licciardello G. *et al.,* 2007; 2009).

1.8. Genome deduced information

1.8.1. Secretion systems

Protein and DNA translocation into neighboring cells and secretion to the extracellular environment is achieved by several secretion/translocation systems in Gram-negative bacteria. These secretion systems have been classified into six types, from the type I secretion system through to the type VI secretion system (T1SS-T6SS) (Tampakaki *et al.,* 2010).

Trantas *et al.,* (2015 a) screened the *P. corrugata* genomes for the evidence of these secretion/translocation systems, using the gene clusters of *P. fluorescens* F113, very closed taxonomically, as sequence queries. Their study evidenced that the 4 *P. corrugata* genomes encode T1SSs, two T2SSs, one T4SS detected only partially, five T5aSSs, three T5bSS, and three T6SSs. Surprisingly, no T3SS-encoding locus was detected in any of the examined strains. A similar results was obtained for the closely related species *P. mediterranea* except for and additional T5SS (T5dSS). The type II secretion system (T2SS) is used by Gram negative bacteria to secrete proteins into the extracellular environment, and it serves as a major virulence mechanism in several bacterial species. It is well-conserved and is composed of a set of 11 to 12 proteins (Whitchurch *et al.,* 2004). *Pseudomonas* T2SSs could be divided in two main clusters, named Xcp (extracellular protein) and Hpx (homolog to Xcp) (Redondo-Nieto *et al.,* 2013). Similar to the closely related species *P. fluorescens* F113, two T2SS-encoded loci, one related to Xcp cluster T2SSs and the other related to Hxc cluster T2SSs, are present in the genome of *Pcor*. The genetic organization of the *Pcor* Hxc cluster is highly similar to the Hxc clusters of *P. fluorescens* F113 and *P. aeruginosa* PAO1 (Ball *et al.,* 2002; Redondo-Nieto *et al.,*

2013). The type III secretion system (T3SS) is a bacterial molecular nano-syringe related to the bacterial flagellum and is required for the translocation of virulence proteins. It is composed of approximately 25 proteins (Tampakaki *et al.,* 2010; Skandalis *et al.,* 2012), and has evolved into seven different families: Ysc, Hrp1, Hrp2, SPI-1, SPI-2, *Rhizobiaceae* and *Chlamydia* (Pallen *et al.,* 2005; Troisfontaines and Cornelis, 2005).

Trantas *et al.* (2015 a) translated and screened the predicted coding sequences from *Pcor* genome with several Hrp/Hrc homologs from *P. syringae*, *P. brassicacearum, Xanthomonas,* and *Erwinia* T3SSs, including a second rhizobial type T3SS cluster and from the 4,469 gene families of *Pcor*, only five that belong to the bacterial flagellum machinery revealed similarity to T3SS. In addition only one T3E-like protein was observed in all examined strains.

The absence of a *Hrp* gene cluster from the genomes of *Pcor* strains is a unique feature of these pathogens, since the majority of the known plant, insect and animal pathogenic pseudomonads rely on the presence of a functional T3SS to manipulate immunity and to colonize their hosts (Tampakaki *et al.,* 2004). The T6SS was initially identified as a protein secretion apparatus involved in virulence of *Vibrio cholerae* on *Dictyostelium* (Mougous *et al.,* 2006) and *P. aeruginosa* on mouse models (Pukatzki *et al.,* 2006). Furthermore, gene clusters coding for the type VI secretion system (T6SS) are present in the majority of plant pathogenic and symbiotic Gram negative bacterial genomes (Sarris *et al.,* 2012), which indicates its importance in the life cycles of these species. Although the exact role of the T6SS in plant colonization is not clear, it is noteworthy that there are multiple T6SS clusters in single strains.

All the *Pcor* predicted proteomes were screened using the T6SS core components ImpB, ImpC and the ClpV1 ATPase of *P. syringae* as sequence queries (Trantas *et al.,* 2015 a). Three independent gene clusters were found. Several genes coding for VgrG (Valine-Glycine Repeat Protein) proteins were found randomly distributed in the genome of both species. This is a common feature of *Pseudomonas* species (Sarris *et al.,* 2010, 2012; Sarris and Scoulica, 2011). Nucleotide alignments of the three T6SS clusters revealed a high degree of similarity between the four sequenced strains of *Pcor* to *P. mediterranea* and *P. brassicacearum* strains.

1.8.2. Antimicrobial peptides and toxins

Trantas *et al.*(2015 a) mined the genome of four *P. corrugata* strains using the software pipeline antiSMASH (Blin *et al.,* 2013) for the automated identification of secondary metabolite biosynthesis clusters in each genome. The analysis identified, in all genomes, a number of non-ribosomal peptide synthetases (NRPS) clusters, a gene cluster related to bacteriocin biosynthesis, a type I PKS cluster a siderophore and an arylpolyene gene cluster. In the *Pcor* CFBP5454 genome at least 10 scaffolds with genes similar to those for syringomycin and syringopeptin biosynthesis, secretion and regulation in *P. syringae* pv. *syringae* strains, thus probably involved in cormycin and corpeptins production and secretion.

BLAST searches of the other NRPS genes mined by antiSMASH suggest that three other different NRPS clusters are present in all strains (Trantas *et al.,* 2015 a). One of them is putatively involved in the production of the lipopeptide siderophore corrugatin, a CLP siderophore (Meyer *et al.,* 2002). BLASTX analysis with the predicted NRPS sequences showed similarity to a putative

siderophore gene cluster in *P. fluorescens* strain SBW25 (Cheng *et al.,* 2013). The SBW25 gene cluster contains five NRPS genes (PFLU3220, 3222–3225) that are predicted to encode an eight-amino acid peptide that resembles ornicorrugatin (similar to corrugatin, but the amino acid 2,4-diaminobutanoic acid is replaced by the amino acid ornithine), a siderophore produced by *P. fluorescens* AF76 (Matthijs *et al.,* 2008).

In addition, a cluster that has high similarity to the achromobactin biosynthesis and utilization cluster of *P. syringae* pv. *syringae* B728a (Psyr2582-2593) and that has been detected in six different *P. syringae* pathovars (Berti and Thomas, 2009) was detected in all *Pcor* strains. They possess highly conserved genes of this cluster, but lack the entire ABC transporter system encoded by cbrA, cbrB, cbrC (Psyr2590-2592).

Typically, *Pseudomonas sensu stricto* produces yellow-green fluorescent pigments when grown under iron-limiting conditions. These fluorescent compounds are pyoverdines that act as siderophores (Cornelis and Matthijs, 2002), which are iron chelating compounds secreted to survive during conditions of iron deprivation. As expected, since *Pcor* is in the non-fluorescent group of the species, no pyoverdine biosynthesis cluster was found.

More bioactive metabolites can be produced by the strains by means of the other two NRPS clusters and by a conserved type I PKS cluster which was not detected in other *Pseudomonas* species deposited in GenBank and IMG databases. Best similarities (approximately 40%) were detected by BLAST within the genomes of the symbiotic bacterium *Rhizobium sullae* wsm1592 and *Mesorhizobium loti* MAFF303099 plasmid pMLa.

In addition, an aryl-polyene (APE) gene cluster was detected in all strains. Recently APE gene clusters were found widely but discontinuously distributed among Gram-negative bacteria (Cimermancic *et al.,* 2014). Aryl polyenes are responsible for yellow pigmentation such as the brominated aryl-polyenes xanthomonadins of *Xanthomonas* species (Cimermancic *et al.,* 2014).

The genomes were also mined by BLAST using the biosynthetic loci of compounds contributing to biological control in other *Pseudomonas* strains such as phenazines, hydrogen cyanide (HCN), pyrrolnitrin, 2,4-diacetylphloroglucinol (DAPG), and pyoluteorin as queries (Loper *et al.,* 2012). Only the hydrogen cyanide gene cluster was found in all *Pcor* genomes (Trantas *et al.,* 2015 a). However, using the Cyantesmo detection card only, the authors observed that only 3 out of the 4 strains produced detectable levels of HCN (Table 3).

The assembled genomes were also queried for genes encoding bacteriocins (Riley and Wertz, 2002), which are antimicrobial peptides produced by bacteria to inhibit the growth of closely related bacterial strains (Hassan *et al.,* 2012). Trantas *et al.,* (2015 a) blasted the translated ORFs of the four genomes under study with antimicrobial peptides from the BAGEL database (Van Heel *et al.,* 2013) and protein hits were recorded detecting in the genomes of the *Pcor* strains genes for the peptides Carocin D and Zoocin A were detected.

Table 3. Features linked with antimicrobial activity of *Pseudomonas corrugata* strains isolated from infected tomato plants used for the comparative analysis (Trantas *et al.*, 2015 a).

Strain	Geographic origin	IMG genome ID	References	Morphology	Hydrolytic activity			HR	HCN	Antimicrobial activity against			
					Casein	Gelatine	Tween 80			*Rp*	*Bm*	*Pst*	*Xcc*
NCPPB2445T	UK	2563366525	NCPPB	R	W	W	+	+	W	+	+	+	−
TEIC1148	Crete, Greece	2563366520	Trantas *et al.*, 2015 a	S	−	−	−	−	−	+	−	−	−
CFBP5403	Tenerife, Spain	2563366523	CFBP	R	+	+	−	+	+	+	+	+	−
CFBP5454	Sicily, Italy	2558309045	Catara *et al.*, 2002	R	+	+	+	+	+	+	+	+	+

Species abbreviations are: Rp, *Rhodotorula pilimanae*; Bm, *Bacillus megaterium*; Pst, *P. syringae* pv. *tomato* and Xcc, *Xanthomonas campestris* pv. *campestris*. TEIC: Technological Educational Institute of Crete Bacterial collection; CFBP: International Center for Microbial Resources, French Collection for Plant-associated Bacteria, INRA, Angers, France; NCPPB, National Collection of Plant Pathogenic Bacteria, Fera, York, U.K. Isolate names followed by the letter "T" are type strains; R, "rough-phenotype" producing yellow diffusible pigments (R-type); S, "smooth phenotype" *with whitish-cream smooth-surfaced colonies that do not produce any pigment (S-type); I, "intermediate phenotype" producing rather smooth colonies with weak diffusible pigment (I-type); HR, hypersensitive response-like reaction; HCN, production of hydrogen cyanide; +, positive reaction; −, negative reaction or no antimicrobial activity; w, weak-positive reaction.*

2. AIM OF THE THESIS

The development of sustainable methods for control of plant diseases has inspired research strategies that have been focusing on: the search for chemical or biological means that can directly interfere with the mechanisms of action of pathogens or to hinder pathogens indirectly by stimulating plant defense responses.

Phytopathogenic bacteria in nature on both spontaneous and cultivated plants have an innate immunity or resistance to infection; these bacteria also do not form particular resistance structures and therefore in theory should be easily controlled. Nonetheless, bacteria are capable of causing serious epidemic plant disease worldwide (Janse, 2006). This is also due to the lack of direct and systemic chemical fighting weapons. Antibiotics in agriculture are banished in most countries and the fight is based on a combination of prevention or protection methods with copper based compounds. The use of both antibiotics, when authorized and copper, involves the evolution of populations of resistant bacteria with other crop damage.

Phytopathogenic bacteria have evolved to interact with the eukaryotes and yet face the surrounding environment and microflora, show a strong adaptation ability, versatile metabolism and ingenious mechanisms of action. All this is done through the production of an arsenal of molecules which in turn are aimed at nutrition, reproduction or survival under adverse conditions. Among secondary metabolites that play a crucial role in molecular interaction between plant and bacterial fitness: linear and cyclic peptides, polycytes, sideropores, indolacetic acid, and esocellular polysaccharides (Mansfield *et al.,* 2012). Interference with

the production, secretion and/or regulation of these secondary metabolites is seen as a potential strategy to fight phytopathogenic bacteria as well as pathogenic bacteria for humans to overcome the criticality of resistance to antibiotics (Koh *et al.,* 2013).

Members of the genus *Pseudomonas sensu stricto* show remarkable metabolic and physiologic versatility, enabling the colonization of diverse terrestrial and aquatic habitats, and are of great interest because their importance in plant and human diseases, and their growing potential in biotechnological applications. They can control plant diseases both indirectly, by competing for space and nutrients or stimulating plant defence resistance response, and directly by producing antibiotic substances. These bacteria may produce several diffusible and/or volatile secondary metabolites with antibiotic properties which inhibit pathogens in vitro. Extensive interest has been devoted to the role of various cyclic lipopeptides (CLP) antibiotics, siderophores, hydrogen cyanide (HCN), 2,4 diacetylphloroglucinol (DAPG), pyrrolnitrin, pyoluteorin, phenazines, in biological control of plant pathogens (Raaijmakers *et al.,* 2006, 2010; Cornellis and Matthijs, 2007).

Model study of this Ph.D. thesis is the bacterium *Pseudomonas corrugata* either described as causal agent of tomato pith necrosis however also as biocontrol agent in a number of pathosystems. *P. corrugata* metabolic versatility makes it attractive for the production of commercial biomolecules and bioremediation (Catara, 2007; Trantas *et al.,* 2015 a). It produces the antimicrobial and phytotoxic cyclic lipopeptides (CLPs) cormycin A, corpeptin A, and corpeptin B (Emauele *et al.,* 1998; Scaloni *et al.,* 2004; Licciardello *et al.,* 2012). The production of these secondary

metabolites seems regulated at high cellular density with a "quorum sensing" mechanism (Licciardello *et al.,* 2007; 2012; Strano *et al.,* 2014). 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. Recently the draft genome sequences of a number of *P. corrugata* strains were obtained opening the opportunity to dissect the mechanisms that *P. corrugata* employs to cause disease, prevent disease caused by other pathogens, and to mine their genomes for genes that encode proteins involved in commercially important chemical pathways (Licciardello *et al.,* 2014a, 2014b; Trantas *et al.,* 2015 a).

The availability of genome sequences represents a revolution in the study of bacteria and their interactions with the surrounding world, enabling many of the mechanisms and evolution to be clarified. Such knowledge is indispensable for the development of new and alternative means of control in agriculture, which must meet the modern needs of reducing the use of agrochemicals and the development of low environmental impact naturally occurring molecules.

Based on previous knowledge already developed for strain CFBP 5454 at the Di3A, University of Catania this thesis project aimed at shed light on:

i. the antimicrobial activity against phytopathogenic fungi and bacteria due to diffusible and volatile compounds of the model strain *P. corrugata* CFBP 5454 and of mutant

bacteria strains unable of producing the CLPs cormicin and corpeptin[1]1;

ii. the targeted search for antimicrobial peptides and other secondary metabolites biosynthetic gene clusters by mining the genome of *P. corrugata* sequenced genomes;

iii. the regulation of secondary metabolite by the AHL-Quorum sensing system by RNAseq analysis of *P. corrugata* mutant strains in two LuxR transcriptional regulators1[2];

iv. the interference of plant natural compounds on bacterial Quorum sensing system and regulated genes.

[1] Results are part of the manuscript: Strano C.P., Bella P., Licciardello G., Caruso A., and Catara V. (2017). Role of secondary metabolites in the biocontrol activity of *Pseudomonas corrugata* and *Pseudomonas mediterranea*. European Journal of Plant Pathology, 149(1): 103-115.

[2] Results have been submitted for publication: Licciardello G. †, Caruso A. †, Bella P., Gheleri R., Strano C.P., Trantas E.A., Sarris P.F., Almeida N.F., and Catara V. PcoR and RfiA, LuxR regulators, co-regulate antimicrobial peptide and alginate production in *Pseudomonas corrugata*. † these authors contribute equally to the work and are co-first authors.

3. MATERIALS AND METHODS

3.1. Bacterial and fungal strains and routine growing conditions

P. corrugata and *P. mediterranea* strains used in this study are listed in Table 4. *Pseudomonas corrugata* strain CFBP5454 and derivative mutants GL2 (*pcoR*-) (Licciardello *et al.,* 2007) and GLRFIA (*rfia*-) (Licciardello *et al.,* 2009), were routinely cultured at 28 °C on either nutrient agar (Oxoid, Milan, Italy) supplemented with 1% D-glucose (NDA), Luria-Bertani (LB) agar (Oxoid, Milan, Italy). 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}.

Table 4. *Pseudomonas corrugata* strains used in this study.

Strains	Genotype/relevant characteristics	Reference or source
CFBP 5454	Wild type	CFBP[a]
GL2	*pcoR*76::Tn5, Km^r	Licciardello *et al.,* 2007
GLRFIA	*rfiA*:: pKnock, Km^r	Licciardello *et al.,* 2009
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

CFBP, International Center for Microbial Resources, French Collection for Plant-associated Bacteria, INRA, Angers, France

The antimicrobial tests were performed against phytopathogenic bacteria and fungi listed in Table 5. The Gram-positive bacterium *Bacillus megaterium* ITM100 and the yeast *Rhodotorula pilimanae* ATCC 26423 were used as bioindicators of cyclic lipopeptide production according to Lavermicocca *et al.,*1997.

Chromobacterium violaceum ATCC 31532, *C. violaceum* CV026 (McClean *et al.,* 1997) were used to evaluate Quorum sensing inhibition activity of plant compounds. Bacteria were routinely grown either on NDA or KB for 24-48 hours according to the species at 28 °C. All bacterial strains were routinely mantained on NDA at 4 °C for short periods and preserved in 15% glycerol at −80 °C for long-term storage. Fungi were routinely grown on Potato Dextrose Agar (PDA; Oxoid, Milan, Italy) at 24 °C and up to one week according to the species. Fungal Cultures were maintained on PDA at 4 °C for short periods and stored on PDA under mineral oil (Sigma-Aldrich, UK) at 4 °C.

Table 5. Bacteria, fungi and biosensor microorganisms used in this study.

Species	Strain	Source	Abbreviation
Phytopathogenic bacteria			
Pseudomonas corrugata	5454	CFBP	Pco
Pseudomonas syringae pv. *Tomato*	28.3.1	PVCT	Psto
Pseudomonas mediterranea	5447	CFBP	Pmed
Pseudomonas chicorii	2101	CFBP	Pch
Xanthomonas campestris pv. *campestris*	568	LMG	Xcc
Xanthomonas vesicatoria	2537	CFBP	Xv
Pectobacterium carotovorum subsp. *carotovorum*	2046	CFBP	Pcc
Erwinia amylovora	683	NCPPB	Ea
Clavibacte r michiganenensis subsp. *michiganensis*	156.1.1	PVCT	Cmm
Phytopathogenic fungi			
Aspergillus niger	22	PVCT	
Penicillum digitatum	235	PVCT	
Botrytis cinerea	BC1	PVCT	
Fusarium oxysporum f.sp. *lycopersici*	Ly1	Saitama	
Phoma tracheiphila	27	PVCT	
Microrganisms used as biosensors			
Chromobacterium violaceum	CV026	McClean *et al.,* 1997	Cv
Chromobacterium violaceum	31532	ATCC	Cv
Bacillus megaterium	100	ITM	Bm
Rhodotorula pilimanae	26423	ATCC	Rp

CFBP, International Center for Microbial Resources, French Collection for Plant-associated Bacteria, INRA, Angers, France; NCPPB, National Collection of Plant Pathogenic Bacteria, Fera, York, U.K; BCCM/ITM, Belgian co-ordinated collections of micro-organism, Mycobacteria Collection, Instituut voor Tropische Geneeskunde, Antwerpen, Belgium; BCCM/LMG, Belgian co-ordinated collections of micro-organism Bacteria Collection Universiteit Gent (UGent), Gent, Belgium; ATCC, American Type Culture Collection, Manassas, Virginia, USA; PVCT, patologia vegetale università di catania, University of Catania, Catania, Italy.

3.2. In vitro antimicrobial activity of *Pseudomonas corrugata*

The production of diffusible antimicrobial compounds was evaluated *in vitro* on PDA and KB medium. To assess the bacterial antagonistic effects on fungal pathogens, small masses of bacterial strains were transferred either onto two or four sides of a Petri dish (1 cm from the edge) containing PDA or KB. Four-mm mycelia discs from a well-grown lawn of PDA cultures of the fungal pathogens were then placed in the middle of the Petri dishes. Plates were incubated at 25 °C and the radius of the fungal colony was measured for up to two weeks. All strains were evaluated in three independent replicates. The antifungal activity was expressed as a Percentage of Growth Inhibition (PGI) according to Vincent (1947): PGI (%) = 100·(GC-GT)/GC; where GC represents the mean value of the fungus radius in the absence of the bacteria and GT represents the mean value of the fungus radius in the presence of antagonistic bacteria.

To assess the antibacterial activity, 10 µl cell suspension drops in sterile distilled water from all the *Pseudomonas* strains ($1*10^8$ CFU mL^{-1}) were spotted onto PDA and KB plates, which had been previously uniformly sprayed with a $1*10^8$ CFU mL^{-1} bacterial suspension of the following strains; *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 diameter of the inhibition zones around the *Pseudomonas* strains was measured.

Hydrogen cyanide (HCN) production was determined qualitatively using Cyantesmo paper (Machery-Nagel GmbH & Co., Düren, Germany). Cyantesmo strips were placed on the lid of LB agar plates seeded with the

Pseudomonas strains and sealed with Parafilm (M). HCN production was evaluated after 24 h of incubation at 28 °C when the strip had changed color from pale green to blue.
Antagonism due to volatile compounds was evaluated based on the effect on *B. cinerea* PVCT-BC1 mycelial growth. *Pseudomonas* strain suspensions (100 µl of 2×10^8 CFU mL^{-1}) were spread onto PDA plates. Separate PDA plates were inoculated either with a four millimeter fungal plug or spread with 100 µl of conidial suspension (1×10^6 conidia mL^{-1}). A double-dish layer was prepared facing the plate inoculated with the bacterial strain and the plate containing the fungal inoculum, thus avoiding direct contact between the two, sharing only the air. The two plates were then sealed with Parafilm (M) and incubated at 25 °C. In the control treatment, no bacteria were seeded in the upper plates. The production of volatile compounds was evaluated either as PGI of fungal radial growth.

3.3. In vitro antimicrobial activity of plant derived compounds

Catechol, citronellol, esperidin, limonene, quercitin and rutin were purchased from Sigma-Aldrich (UK) and were dissolved in DMSO. Antimicrobial activity was tested using the disc diffusion method. Nutrient Dextrose agar plates were seeded with Gram-negative and Gram-positive phytopathogenic bacteria, with a sterile cotton swab soaked with a bacterial suspension obtained with colonies from a fresh overnight NDA culture diluted in sterile water at the standard concentration of $1*10^8$ CFU mL^{-1} using a spectrophotometer (Eppendorf Biophotometer)

(OD600=0.1). Then sterile filter paper discs (6 mm in diameter) (Oxoid, Milan, Italy) were placed on seeded plates by a Disc-dispenser (Oxoid, Milan, Italy) and were impregnated with 10 µl of 100 mg mL^{-1} of either cathecol, citronellol, esperidin, limonene, quercitin or rutin and incubated at 28 °C for 24-48 hours. The equivalent volume of solvent (DMSO) served as negative control. Gentamicin (10 µg $disc^{-1}$) (Oxoid, Milan, Italy) was used as positive control. All experiments were performed in triplicate.
Microbial growth in liquid media added with plant compounds was automatically determined using a Bioscreen C (Labsystems, Helsinki, Finland) (by the broth dilution method), an automated turbidimeter that measures kinetically, the development of turbidity. Bacteria culture of all strains were grown overnight in LB picking a single colony from a fresh overnight NDA culture, and normalized to $1*10^8$ CFU mL^{-1} with fresh liquid LB as described above. Aliquot of 198 µl of inoculated LB where dispensed in each well of a microplate and then where added 2 µl of each tested compounds (2-fold serial dilutions). The MIC was determinated as the lowest concentrations of the compound at which no measurable growth (increase in turbidity) occurred for a given strain. Each experiment was performed at least twice with three replicates for each strain/compound combination.

3.4. Quorum quenching activity of plant natural compounds

The reporter strain *Chromobacterium violaceum* ATCC 31532 was used as biosensor to evaluate the potential Quorum sensing-inihibition of catechol, citronellol,

esperidin, limonene, quercitin and rutin using the disc diffusion method as described above. In this bacteria the production of violacein is regulated by AHL-QS, and his production is easily detected due to the purple color of colonies. After 24 hours at 28 °C the presence of white colonies near the tested compound was recorded as QS-I.
In order to determine the quorum quencing activity of citronellol against medium chain AHLs (C6-HSL and C8-HSL) we also used the QS *C. violaceum* CV026 (McClean *et al.,* 1997) the reporter strain commonly used to detect quorum sensing activity. CV026 is a white colony mutant that could be used as biosensor able to respond to a series of synthetic AHLs which induce violacein production, this change could be inhibited in presence of QQ compounds. LB agar plates were inoculated with a sterile cotton swab, with colonies from a fresh overnight LA culture of *C. violaceum* CV026 (OD600 0.5) diluted in sterile water at the standard concentration corresponding to a 0.5 OD600 using a spectrophotometer (Eppendorf Biophotometer). Wells were made in the agar plates and filled with 20 µl of 200 µM concentration of C6-HSL and C8-HSL and 10 µl of citronellol at different concentrations in DMSO (50, 25 and 12.5 mg mL^{-1}) and DMSO was used as control. After 24 hours of incubation at 28 °C the presence of white colonies near the wells was recorded as QS-I. AHLs used in this work were purchased from Sigma-Aldrich (UK) and were dissolved in ethyl acetate.

3.5. β-Galactosidase assay *P. corrugata*

β-Galactosidase activity was determined to analize pcoI promoter activity in WT (*pMPPcoI*) and GL1 (*pMPPcoI*) (Licciardello *et al.,* 2009) strains harboring a pcoI-lacZ

reporter construct, during growth in LB medium in presence of exogenous AHLs and citronellol. Transcriptional fusion based on the pMP220 promoter probe vector was constructed by cloning the 695-bp fragment containing the pcoI promoter region in the corresponding sites in pMP220, yielding pMPPcoI. The plasmid was then trasferred by triparental conjugation into the *P. corrugata* CFBP 5454 Wt strain and in the GL1 (*pcoI*) mutant derivative (Licciardello *et al.,* 2009).

Overnight cultures in LB medium were diluted to OD600 nm 0.01 (at initial inoculum of 10^8 CFU mL^{-1}) in 10 mL of fresh LB with 100 µl of citronellol at different concentrations (12.5, 25, 50 mg mL^{-1}) and in presence or absence of C6-AHL (5 µM), as shown in the list below, and incubated at 28 °C with shaking at 180 rpm. DMSO, used to resuspend citronellol, was added as control. Tetracycline at 50 µg mL^{-1} concentration was added to the cultures.

After 24 hrs of incubation, β -galactosidase activity was measured as described by Miller (1972), with the modifications of Stachel *et al.* (1985). All experiments were performed in triplicate.

- WT (*pMPPcoI*) / GL1 (*pMPPcoI*);
- WT (*pMPPcoI*) / GL1 (*pMPPcoI*) + C6-AHL;
- WT (*pMPPcoI*) / GL1 (*pMPPcoI*) + Citronellol (50 mg mL^{-1});
- WT (*pMPPcoI*) / GL1 (*pMPPcoI*) + C6-AHL + Citronellol (25 mg mL^{-1});
- WT (*pMPPcoI*) / GL1 (*pMPPcoI*) + C6-AHL + Citronellol (12.5 mg mL^{-1});
- WT (*pMPPcoI*) / GL1 (*pMPPcoI*) + C6-AHL +DMSO.

β -galactosidase activity, measured in Miller Units, has been calculated with the following formula: OD420 x 10^3 /

OD600 x T, where T is the time expressed in minutes, OD420 absorbance at 420 nm and OD600 absorbance at 600 nm.

3.6. In vitro bioassay for CLP production

P. corrugata strains were grown either in IMM (Surico *et al.,*1988) up to 4 days after inoculation depending on the trials or in LB for 24 hrs at 28 °C in static conditions. Bacterial cultures were centrifuged at 9000*g for 20 min to eliminate cells. The supernatant was passed through a 0.25 µm Millipore filter (Millipore, Billerica, MA, U.S.A.) to obtain cell-free culture filtrates. Aliquots of all samples were lyophilized and re-hydrated in sterile water to obtain a 10X culture filtrates. Cultural filtrates of *P. corrugata* and mutant derivatives were evaluated for CLPs production by a bioassay based on antimicrobial activity against *R. pilimanae* ATTC 26432, *B. megaterium* ITM100, performed essentially as previously described (Licciardello *et al.,* 2009). Wells (6 mm Ø) were made in the PDA using a cork borer, and 10 µl of CF were spotted onto each well. The plates were incubated for up to four days at 27 °C. The inhibition halos were measured starting from the edge of the well. Each test was carried out twice in triplicate.

3.7. In vivo assays

In vivo assays were performed to evaluate *P. corrugata* CFBP 5454 and derivative mutants listed in table 4 for biocontrol activity against *Penicillum digitatum* in lemon fruits and to verify strain plant interaction-phenotypes.

3.7.1. Biocontrol assays

For Biocontrol assays lemons from organic farming 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 for each strain were used. Drops of 20 µl of bacterial suspensions ($2*10^9$ CFU mL^{-1}) were pipetted into each wound and allowed to dry. Subsequently, the wounds were inoculated with 10 µl of conidial suspension $1*10^6$ 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 and using a disease index based on four values: DI 0= no symptoms; DI 1= presence of rot tissues; DI 2= presence of mycelium; DI 3= presence of sporulation.

3.7.2. Pathogenicity assays

P. corrugata CFBP 5454 and derivative mutants were tested for pathogenicity on tomato cv. Bacio plants grown in nursery flats, 1 month after germination. During the trials, plants were maintained in a growth chamber with a 16 h/8 h photoperiod and a temperature of 26 °C. Tomato plants were pin-pricked on the stem at the axil of the first true leaf with bacterial cells from 48-h culture on NDA (Licciardello *et al.,* 2007). The length of the stem discoloration/necrosis was assessed at 15 dpi. An HR test was performed by infiltration of *N. benthamiana* leaf mesophyll with a bacterial suspension of 10^8 CFU mL^{-1} using a blunt syringe. Twenty leaf panels were inoculated per strain. After

inoculation, plants were placed at 25 °C in a growth chamber and the collapse/necrosis of the mesophyll was recorded daily (Strano *et al.,* 2015)

3.8. EPS isolation and quantification

Bacterial cells obtained as described above were removed from the culture broth by centrifugation at 16,300 g for 20-30 min. The supernatant was boiled for 15 min to inactivate any possible depolymerase and then centrifuged at 1,700 g for 30 min at 4 °C. EPS have been isolated according to Fett *et al.* (1996) with slight modifications. A concentrated aqueous solution of KCl (25% w/v) was added to the supernatant at a final concentration of 1% (w/v) to aid in the precipitation. EPS were then precipitated by the addition of 3 volumes of isopropanol, sitted at 4 °C collected by centrifugation (1,700 g, 20 min) and redissolved in distilled water. Precipitation with isopropanol was repeated three times and final pellet taken up in Milli-Q purified water, freeze-dried and weighed. Three separate partially purified samples were prepared for each bacterial strain.

3.9. Statistical analysis

Data were analyzed depending on the experiment either by two-way ANOVA using IBM® SPSS® 20 (antagonisitic activity of *Pseudomonas* strains) or using STATGRAPHICS Plus 5 (in vivo test). Mean values were compared using the Student–Newman–Keuls test. Interaction between the study variables was also investigated. Statistical significance was established at $P \leq 0.05$ and $P \leq 0.001$.

3.10. Transcriptomic studies

3.10.1. RNA isolation

RNA was obtained from bacterial samples for different purposes as follow:

- Transcriptomics: RNA samples were extracted from *P. corrugata* CFBP5454 and derivative mutants 40 hours after inoculation, at the early stationary phase (OD600 of 8.9). The three biological replicates of each strain were extracted on separate days with separate batches of media (IMM, Surico *et al.,* 1998);
- Validation of RNA-Seq results: in order to validate the RNA-Seq results, RNA samples were extracted from *P. corrugata* CFBP5454 and derivative mutants cultures at the same conditions described above;
- Expression in rich medium: for studying differential gene expression between rich and minimal medium, RNA samples were extracted from *P. corrugata* CFBP5454 NB cultures at the early stationary phase;
- Effect of citronellol to analyze the effect of citronellol on the expression of *P. corrugata* Quorum sensing regulated genes, was grown in 20 ml of IMM (Surico *et al.,* 1998) added with 200 µl of citronellol at different concentration (50, 25 and 12.5 mg mL^{-1}) at 28 °C for 4 days in static conditions.

Bacterial cultures were fixed using RNA protect TM Bacterial Reagent (Qiagen) in a ratio of 2 mL of reagent per 1 mL of bacterial culture. Centrifugation was used to pellet

the cells (5000 rpm, 4 °C, 20 min) and the supernatant was discarded. Samples were extracted using an RNeasy Mini Kit (Qiagen) and eluted in RNase/DNase free water. Total RNA samples were quantified using micro-spectrophotometry (NanodropTM2000C, Thermo ScientificTM, Waltham, Massachusetts, USA).The RNA quality was measured using an Agilent 2100 Bioanalyzer and only RNA samples with an RNA Integrity Number (RIN) above 8.0 were selected.

3.10.2. RNA isolation from inoculated plants

RNA was extracted from tomato cv. Bacio plants previously inoculated with *Pseudomonas corrugata* CFBP 5454. Tomato plants were grown in nursery flats, after germination and during the trials, were maintained in a growth chamber with a 16 h/8 h photoperiod and a temperature of 26 °C. Tomato plants were pin-pricked on the stem at the axil of the first true leaf with bacterial cells from 48-h culture on NDA (Licciardello *et al.,* 2007). Four days after inoculation, 5 cm of stem including the inoculation site was cut and stored at -80°C. Pools of four stems for each bacteria-inoculated plant, were grounded in liquid nitrogen and 100 mg of powder processed for total RNA extraction by Rneasy Plant minikit (Qiagen Inc.), according to manufacturer's instruction.

3.10.3. Transcriptome analysis by RNAseq

Libraries were prepared for sequencing according to the manufacturer's instructions (Illumina). Single-end 51 nucleotide sequence reads were obtained using the Illumina HiSeq2000 system at Parco Tecnologico Padano (Lodi, Italia), processed with Casava version 1.8. Raw sequencing reads were quality controlled using FastQC v.0.10.1 and

processed with Trimmomatic v.0.32 to remove sequencing adapters and low quality bases. High quality filtered reads were aligned against the draft genomes of *P. corrugata* (ATKI01000000). The Bowtie v2.2.2 software was used to perform the alignments and generate the corresponding BAM files.

The draft genome of *P. corrugata* CFBP5454 was re-annotated using PROKKA v1.9 to generate a set of predicted genes and their relative position on the scaffolds, using core bacterial genes and the SwissProt dataset as reference. Aligned reads were processed using HTSeq v0.6.1 to extract reads counts over the annotated genes for the genome provided. For all samples, the number of raw reads mapping to each gene was normalized based on the total number of input reads (non-rRNA and non-tRNA reads) for that sample. This normalization procedure allowed comparison of gene-expression patterns across strains, within and between experiments. Reads that partially overlapped a gene contributed to its total raw read value. Next we applied filtering criteria designed to allow a more robust estimation of the quorum-activated component of the quorum regulon. Only genes that had an average of >10 reads in the three replicates for the two mutants in comparison to Wt were considered for further analyses. The reads counts for each sample were imported into R and processed using the Bioconductor package EdgeR. Counts values were normalized using the TMM method (Trimmed Mean of M-values) and statistical comparisons of expression levels across different groups were performed using the EdgeR exact test method.

For our further analyses genes with false discovery rates = 0.05 were selected. We chose to rely on the top 243

differentially expressed genes without any limitation of fold change.

3.10.4. Quantitative Real-time PCR

cDNA synthesis

Following a DNAse purification step by DNAse I (Invitrogen, Life technologies, Italy) one microgram of RNA was used for cDNA synthesis with Superscript III (Invitrogen, Life technologies, Italy) according to the manufacturer's protocol.

cDNA was synthesized from two nanograms of initial RNA (previously quantified using the spectrophotometer Nanodrop 1000 - Thermo Fisher Scientific) in 20 microliters of reaction. Initially, was prepared a 13 microlitre reaction mixture containing 100 ng of random examers and dntp mix 0.5 mM, were incubated at 65 °C for 5 minutes and then transferred to ice for one minute. Subsequently a second reaction mixture containing the First Buffer 1x, DDT 5mM, the reverse transcriptase SuperScript III RT 1U (Invitrogen, Life technologies, Italy) was added. The reaction was incubated for 5 min at 25 °C, and then at 55 °C for 60 minutes. The transcription reaction was then blocked by incubating at 70 °C for 15 min. To eliminate non-transcribed RNA, RNase H (2U) treatment was performed by incubating the cDNA containing mixture at 37 °C for 20 min. 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.

Primer design

For validation of RNAseq by quantitative RT-PCR, genes belonging to the CLPs and alginate genetic clusters among those more differentially expressed in the WT in comparison

to the two mutants, were selected. A total of 13 genes involved in CLPs production and secretion have been selected (*crpC*, *grsb_1*, *grsB_2*, *dhbF_3*, *dhbF_4*, *syrD2*, *bepE_1*, *mefA*, *ArpC*, *crpD*, *pcoA*, *pcoB*, *oprM_3*). Three genes (*algD*, *algG*, *algI*) belonging to the biosynthetic cluster of alginate were also used for validation.
Nucleotide fasta sequences have been retrieved from the P. *corrugata* CFBP5454 genome (ATKI01000000) and used to design the primer sets useful for qRT-PCR. Primer were designed from the sequence by using Beacon design software (Premier Biosoft International Ltd., Palo Alto, CA, USA), and blasting the sequence obtained to verify the absence of correspondence of other genome sequences.

Quantitative RT-PCR (qRT-PCR)

Reactions were conducted with the BioRad iQ Cycler and the SYBR® Select Master Mix for CFX (Applied Biosystem, Life Technologies) according to the manufacturer's protocols. To correct small differences in template concentration, the *16S rRNA* gene was used as reference control gene (Conte *et al.,* 2006). The cycle where the SYBR green fluorescence crosses a manually set threshold cycle (CT) was used to determine transcript levels. Primers used for these reactions are listed in Table 6; Pco16S sense/16S anti-sense being utilized for normalization (Conte *et al.,* 2006). Analysis of the dissociation curve ensured that a single product was amplified. Cycling consisted of 95 °C for 15 sec, 58-64 °C for 1 min (for annealing temperatures see table 6). Data were analyzed using the comparative Ct method, wherein the Ct values of the samples of interest are compared to the Ct values of a control. All the Ct values were normalized to endogenously expressed genes, in this case the 16S

ribosomal RNA gene. The relative expression (RE) values were calculated by the formula RE = 2-[ΔCT(Wt)-ΔCT(mutant)] (Livak and Schmittgen, 2001). QRT-PCR analysis was performed in duplicate on three independent RNA isolations.

Table 6. Oligonucleotides used in this study

Gene	Sequences	T annealing °C	References
mefA-fw	5'-CAGTGTGTTTGCCTTGAT-3'	59	This study
mefA-rew	5'-GAACCAATGATCGACCAG-3'	59	This study
ArpC-fw	5'-CGCAAGACCTACAAGGAACA-3'	59	This study
ArpC-rew	5'-CGCCAGCCTGTAGTAGTC-3'	59	This study
oprM_3-fw	5'-CCATTCAGTACAAGGAAGG-3'	56	This study
oprM_3-rew	5'-CATCTTCGGCATTGAGTC-3'	56	This study
bepE_1-fw	5'-ATTCTCAACGTCAAGGAT-3'	56	This study
bepE_1-rew	5'-AGCGAGTAATCACCAATA-3'	56	This study
grsB_1-fw	5'-AGAGACCGTGCAGAACAT-3'	59	This study
grsB_1-rew	5'-CGATGGATTGCCGTGAAT-3'	59	This study
grsB_2-fw	5'-CTGAACTATCGTCACCTT-3'	59	This study
grsB_2-rew	5'-CAGAACCTGGATACCTTC-3'	59	This study
dhbF_3-fw	5'-CGTCAGTCGCCTATTGGA-3'	59	This study
dhbF_3-rew	5'-GAAGCCGATGGAGTGGAA-3'	59	This study
dhbF_4-fw	5'-TGAAACCGAAATCAATCTG-3'	56	This study
dhbF_4-rew	5'-CGAAGAAATGATCGTGAC-3'	56	This study
AlgD-fw	5'-CGAAAGCCCACTGGTAGAAC-3'	58	This study
AlgD-rew	5'-AAGTCGGAGTTGAGCAAGGA-3'	58	This study
AlgI-fw	5'-GGCTTCATCAAGAAAGTGTTC-3'	58	This study
AlgI-rew	5'-ATGGACTGGCTGATGTAGGG-3'	58	This study
AlgG-fw	5'-GTTCTCGGACATGTGGTACGG-3'	58	This study
AlgG-rew	5'-GAAATGATGATCCCGTGCTT-3'	58	This study
syrD_2-fw	5'-CAACTGAACACGACACTG-3'	56	This study
syrD_2-rew	5'-GTAGGCAATACCGAACAG-3'	56	This study
macA-fw	5'-CAAAATCGCTATGCTGCTTGTC-3'	60	Strano *et al.,* 2017
macA-rew	5'-CGACCGTAGCGGTCAGGTA-3'	60	Strano *et al.,* 2017
Ribpepsynt (ppsE)-fw	5'-ACGGGCCACCCGAAAG-3'	60	Strano *et al.,* 2017
Ribpepsynt (ppsE)-rew	5'-GAGGCGAAAGCCACGTGAT-3'	60	Strano *et al.,* 2017
Pco16s-fw	5'-TGTAGCGGTGAAATGCGTAGAT-3'	58	Conte *et al.,* 2006
Pco16s-rew	5'-CCTCAGTGTCAGTATCAGTCCAG-3'	58	Conte *et al.,* 2006

3.11. Bioinformatics tools for genomic and transcriptomic data

Analysis for sequence similarity search of genomic data were performed using the BLAST sequence analysis tool (Basic Local Alignment Search Tool) (Altschul *et al.,* 1997) which uses heuristics to produce results quickly. The BLASTN nucleotide-nucleotide search looking for more distant sequences and BLASTX searching a nucleotide query against a protein database, translating the query on the fly, have been used. BLAST queries were accomplished also at Joint Genome Institute (JGI) (Mavromatis *et al.,* 2009).
Genome comparative analysis and gene cluster visualization have been performed using the Integrated Microbial Genomes & Microbiomes (IMG/M, https://img.jgi.doe.gov/) system which provide scientists worldwide free support for genome & microbiome data annotation & integration and open access comparative analysis of integrated genome and microbiome datasets.
The software pipeline antiSMASH (Blin *et al.,* 2013) has been used for the automated identification of secondary metabolite biosynthesis clusters in the *P. corrugata* CFBP5454 genome in comparison to other *P. corrugata* strains.
The phylogenetic trees were generated in MEGA6 (Tamura *et al.,* 2013). Neighbor-Joining trees were generated according to the p-distance method (Saitou and Nei, 1987). The percentage of replicate trees (1500 replicates) in which the associated strains clustered together was estimated (Felsenstein, 1985) and is shown next to the tree nodes.
The number of genes upregulated or downregulated in the WT in comparisons to GL2 and GLRFIA mutants have been showed by Venn diagram using the online tool VENNY 2.1

available at the URL http://bioinfogp.cnb.csic.es/tools/venny/index.html, designed by Oliveros J.C. (2007-2015). Graphical representation of the relationship between intensity (LogCPM) and difference (Log2FC) of genes between *P. corrugata* CFBP5454 (WT) vs GL2 and GLRFIA derivatives mutants has been done using DEPICTViz, Differential Expression and Protein InteraCTions Visualization tool (http://jau.facom.ufms.br/Pcor/). In the plot red points represent genes that were classified as differentially expressed (FDR ≤0.05). Points along the x-axis represents the average intensity of gene expression between conditions. The points above '0' on the y-axis represent genes that are more expressed in WT condition than in GL2 or GLRFIA. Points below '0' on the y-axis represent genes that are more expressed in GL2 or GLRFIA condition than in WT. Functional annotation which allows categorization of genes in functional classes has been performed manually by Gene ontology (GO) identification developed at the GO Consortium (Ashburner *et al.,* 2000) available at the web site http://www.geneontology.org/.

4. RESULTS AND DISCUSSION

4.1.Mutational phenotypes of *Pseudomonas corrugata* CFBP 5454

4.1.1. Diffusible compound-mediated antimicrobial activity

P. corrugata strain CFBP 5454 was assayed *in vitro* against different plant pathogenic fungi and bacteria to investigate the antimicrobial potential due to the production of diffusible compounds. *P. corrugata* in culture produces the antimicrobial phytotoxic and surfactant cyclic lipopeptides (CLPs) corpeptins and cormycin (Licciardello *et al.,* 2012).
In previous studies, we demonstrated that mutants in the LuxR transcriptional regulators PcoR (GL2 mutant) and RfiA (GLRFIA mutant) were impaired in CLP production (Licciardello *et al.,* 2012). Thus, the above-cited mutants were also tested in order to focus on the role of CLPs and to investigate the presence of other antimicrobial compounds. *P. corrugata* strain CFBP 5454 produced diffusible compounds that were able to inhibit the *in vitro* growth of the plant pathogenic fungi *A. niger*, *B. cinerea* PVCT-BC1, *P. tracheiphila* PVCT27, *F. oxysporum* f.sp. *lycopersici* (Saitama ly1), and *P. digitatum* PVCT235. Similar results were obtained on both PDA and KB medium, except for *B. cinerea* PVCT-BC1 and *P. tracheiphila* PVCT27 which were not able to grow on KB. Five day post-inoculation on PDA, strain CFBP 5454 inhibited mycelial growth, with PGI ranging from 38% to 56% compared to the control plates containing only the target fungus, respectively (Table 7). On KB, PGI of approximately 55%, 40%, 32% against *A. niger*, *P. digitatum* PVCT235 and *F. oxysporum* f.sp.

lycopersici (Saitama ly1), for the two strains were observed, respectively (Table 8). The mutant strains behaved differently. On PDA, mutant strain GLRFIA (*P. corrugata* rfiA mutant) showed a significantly reduced antagonistic activity compared to its respective parental strain ($P < 0.001$). GL2 (*pcoR* mutant) strain did not differ from its parental strain CFBP 5454 or from complemented strains of all mutants (Table 7).

On KB transcriptional regulator mutant strains i.e. *P. corrugata* GL2 (pcoR mutant) and GLRFIA (rfiA mutant) showed a statistically significant ($P < 0.001$) reduction in antifungal activity compared to the parental strain CFBP 5454 (Table 8). The inhibitory activity was restored in complemented strains. Fifteen days after plate inoculation, on both culture media, mycelia of *A. niger*, *B. cinerea* PVCT-BC1, *P. digitatum* PVCT235 had overgrown the GLRFIA (*P. corrugata* rfiA mutant) bacterial spot.

A time course experiment was conducted *in vitro* against the fungus *Penicillium digitatum* fungus analyzing the percentage reduction in radial growth of *P. digitatum* induced by the bacteria. The results were recorded time course every 24 hours for up to eight days after inoculation. Comparing the radial growth of the fungus, it can be seen that three days after inoculation *P. digitatum* cultures show a marked growth decrease compared to the control plates with only the fungus (average radius 2.8 cm) (Fig. 4, 5). It is also apparent that the all bacteria at that date reduced fungal growth (medium radius from 1.6 cm for GLRFIA strain to 1.2 cm for CFBP 5454 strain). Four days after inoculation PGI range from 40.5% in the presence of the GL2 strain to 57.5% with the CFBP 5454 parent strain (Fig. 4, 5). In the subsequent observation period, the data showed a stabilization of *P. digitatum* cultures in plates containing

CFBP 5454, GL2, GL2C and GLRFIAC bacteria which at the end of the test reduced the radial growth of the fungus by 68%, 62%, 63.5% and 65% respectively (Fig. 4, 5). From the records carried out six days after inoculation, a resumption of radial growth of *P. digitatum* was observed in the plates with the GLRFIA (rfiA-) strain, which gradually came to cover bacterial spots (Fig. 4). It should be noted that the activity of the complemented strain of the rfiA gene, GLRFIAC, was identical to that of the parental strain CFBP 5454 to demonstrate that this regulator is involved in the antagonistic activity of *P. corrugata*. At last, eight days after inoculation, the percentage of inhibition of the radial growth of *P. digitatum* was similar to the parental strain CFBP 5454, the GL2 mutant and the two complementary strains (about 65%) while inhibition GLRF-induced rate was 37%, as shown in Figure 5. Statistical analysis, revealed that CFBP 5454 wild strain, GL2 mutant strain, and complemented GL2C and GLRFIAC strains exhibit high antifungal activity significantly similar between them, while the GLRFIA mutant strain differs significantly from the other strains (data not shown).

When tested against phytopathogenic bacteria, on PDA *P. corrugata* strain CFBP 5454 showed antimicrobial activity against the Gram-positive bacterium *C. michiganensis* subsp. *michiganensis* PVCT 156.1.1 and to a lesser extent against the Gram-negative *P. syringae* pv. *tomato* PVCT 28.3.1 and *X. campestris* pv. *campestris* PVCT 62.4 (Table 9). On KB *P. corrugata* showed activity only against *C. michiganensis* subsp. *michiganensis* PVCT 156.1.1 (data not shown). Mutational analysis of the antagonistic activity did not reveal any significant differences between mutants in either of the transcriptional regulators and its respective parental strain (Table 9).

4.1.2. Antimicrobial activity of cell-free culture filtrates

P. corrugata strain CFBP 5454, as well as its cell-free culture filtrate, showed antimicrobial activity against the two CLP bioindicators, the yeast *Rhodotorula pilimanae* ATCC26423 and the Gram-positive bacterium *Bacillus megaterium* ITM100 (Table 10). Mutant strain GLRFIA(*rfiA* mutant) did not show antimicrobial activity againstthe yeast *R. pilimanae* ATCC26423, but was still able to inhibit the growth of the bacterium B. megaterium ITM100. *P. corrugata* GL2 (*pcoR* mutant) strain retained the antimicrobial activity against both bioindicators (Table 10). Antimicrobial activity of the cell-free culture filtrates of all mutants (i.e. GL2, GLRFIA) was abolished (Table 10).

4.1.3. Antifungal activity mediated by volatiles

P. corrugata strain CFBP 5454 bacterial volatiles were able to inhibit the growth of *B. cinerea* PVCT-BC1 (PGI of 60%). In addition, the fungal colonies exposed to the bacterium was white, whereas of the colonies on the plates containing only the fungus showed a typical grey conidial sporulation (grey mold). The microscope analysis confirmed the absence of conidia in the white colonies. The mutants of both transcriptional regulators impaired in CLP production GL2 (pcoR mutant) and GLRFIA (*P. corrugata rfiA* mutant) were still able to produce HCN (data not shown). All bacterial strains produced HCN as demonstrated by the color change of the Cyantesmo test strips in response to the detection of the liberated hydrocyanic acid (HCN) gas.

Table 7. Growth inhibition of phytopathogenic fungi due to diffusible compounds produced by *Pseudomonas* spp. strains on potato dextrose agar medium (PDA).

		Percentage of fungal growth inhibition (PGI) [§] [*]					
	Strain genotype	**AN**	**BC**	**FOL**	**PD**	**PT**	**Average PGI/strain†**
CFBP 5454	Wild type	56.25 ± 1.80	52.63 ± 1.52	40.16 ± 0.93	56.06 ± 2	37.98 ± 4.10	48.61 b
GL2	pcoR mutant	55.20 ± 1.04	53.50 ± 0.87	41.66 ± 0.39	54.54 ± 1.31	30.23 ± 2.68	47.02 b
GLRFIA	rfiA mutant	44.8 ± 1.04	28.95 ± 1.52	34.08 ± 1.16	43.18 ± 1.31	22.48 ± 1.55	34.69 a
GL2C	GL2 complemented	52.1 ± 1.04	51.75 ± 0.87	35.18 ± 0.92	53.03 ± 3	30.23 ± 2.68	44.45 b
GLRFIAC	GLRFIA complemented	54.16 ± 1.04	51.75 ± 2.32	37.96 ± 0.92	52.27 ± 1.31	34.88 ± 2.68	46.20 b

[§] AN: *Aspergillus niger*; BC: *Botrytis cinerea* PVCT-BC1; FOL: *Fusarium oxysporum* f.sp. *lycopersici* (Saitama ly1); PD: *Penicillum digitatum* PVCT235; PT: *Phoma tracheiphila* PVCT27.

*Values are means ± standard error (SE).
†Averages followed by the same letter within columns are not significantly different according to Student–Newman–Keuls test (P = 0.001). The results presented are representative of two independent experiments.

Table 8. Growth inhibition of phytopathogenic fungi due to diffusible compounds produced by *Pseudomonas* spp. strains on King's B agar medium (KB).

	Percentage of fungal growth inhibition (PGI) §*				
	Strain Genotype	**AN**	**FOL**	**PD**	**Average PGI/strain †**
CFBP 5454	Wild type	55.30 ± 0.75	31.85 ± 0.74	39.56 ± 0.94	42.24 c
GL2	pcoR mutant	29.54 ± 2.62	21.48 ± 0.7	44.28 ± 0.94	31.70 b
GLRFIA	rfiA mutant	18.18 ± 3.93	23.70 ± 0.7	39.56 ± 0.94	27.15 a
GL2C	GL2 complemented	54.54 ± 1.31	27.40 ± 0.74	45.23 ± 0.9	42.39 c
GLRFIAC	GLRFIA complemented	54.54	32.59 ± 0.74	37.67 ± 1.63	41.60 c

§AN:*Aspergillus niger*; BC *Botrytis cinerea* PVCT-BC1; FOL: *Fusarium oxysporum* f.sp. *lycopersici* (Saitama ly1); PD: *Penicillum digitatum* PVCT235.

*Values are means ± standard error (SE).
†Averages followed by the same letter within columns are not significantly different according to Student–Newman–Keuls test (P = 0.001). The results presented are representative of two independent experiments.

Table 9. Antimicrobial activity due to diffusible compounds produced by *Pseudomonas* spp. strains on potato dextrose agar medium (PDA) against phytopathogenic bacteria.

		Bacterial inhibition zone (mm)[§] *			
	Strain Genotype	**PST**	**CMM**	**XCC**	**Average PGI/strain †**
CFBP 5454	Wild type	8.66 ± 0.33	24.33 ± 0.33	6.66 ± 1.20	13.22 b
GL2	pcoR mutant	11.66 ± 1.76	16.66 ± 0.33	3.33 ± 0.33	10.55 ab
GLRFIA	rfiA mutant	7.66 ± 0.66	17.66 ± 0.33	4.83 ± 0.83	10.05 a
GL2C	GL2 complemented	10.66 ± 0.66	19.33 ± 0.88	4.66 ± 0.66	11.55 ab
GLRFIAC	GLRFIA complemented	8.33 ± 1.20	19.66 ± 0.88	5.66 ± 0.88	11.22 ab

[§]PST: *Pseudomonas syringae* pv. *tomato* PVCT 28.3.1; CMM: *Clavibacter michiganensis* subsp. *michiganensis* PVCT 156.1.1; XCC: *Xanthomonas campestris* pv. *campestris* PVCT 62.4.

*Values are means ± standard error (SE).
†Averages followed by the same letter within columns are not significantly different according to Student–Newman–Keuls test (P = 0.001). The results presented are representative of two independent experiments.

Table 10. Antimicrobial activity of *Pseudomonas* strains and their culture filtrates on potato dextrose agar medium (PDA) against CLP bioindicator strains *Rhodotorula pilimanae* ATCC26423 and *Bacillus megaterium* ITM100

		Strain inhibition zone (mm) *			Cultural filtrate inhibition zone (mm) *		
	Strains Genotype	***R. pilimanae***	***B. megaterium***	**†Average**	***R. pilimanae***	***B. megaterium***	**†Average**
CFBP 5454	Wild type	7 ± 0.57	7.33±0.33	7.16 b	6.66 ± 0.33	5.66 ± 0.33	6.16 b
GL2	pcoR mutant	7.33 ± 0.33	7.33 ± 0.33	7.33 b	0	0	0 a
GLRFIA	rfiA mutant	0	8 ± 0.57	4 a	0	0	0 a
GL2C	GL2 complemented	7 ± 0.57	6.66 ± 0.33	6.83 b	6.33 ± 0.33	5.86 ± 0.46	6.1 b
GLRFIAC	GLRFIA complemented	7.33 ± 0.66	7 ± 0.57	7.16 b	6.66 ±0.33	5.83 ± 0.16	6.25 b

A two-way analysis of variance (ANOVA) was performed to investigate differences in the CLP antimicrobial activity of *Pseudomonas* spp. bacterial cells and cultural filtrates.

*Values are means ± standard error (SE).

†Averages followed by the same letter within columns are not significantly different according to Student–Newman–Keuls test (P = 0.001). The results presented are representative of two independent experiments.

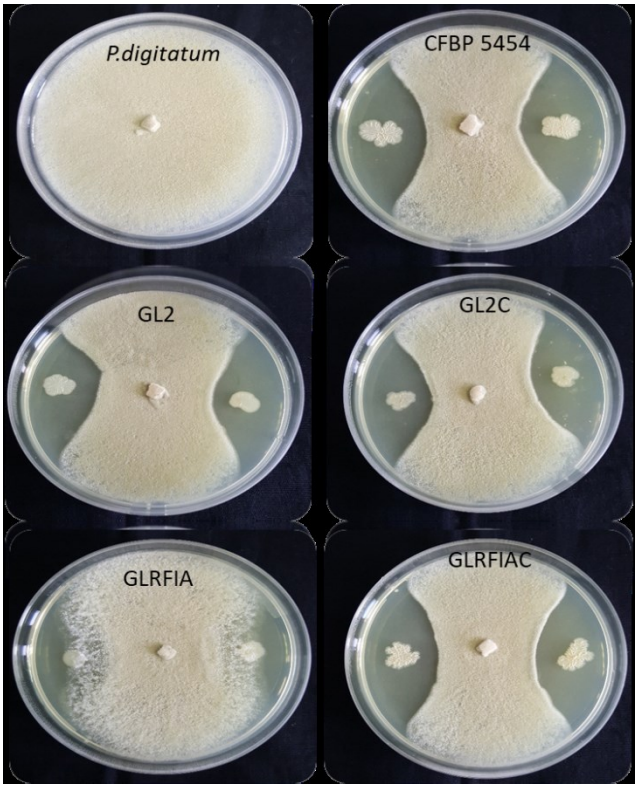

Figure 4. Evaluation of the *in vitro* antagonistic activity of *P. corrugata* CFBP 5454 parental strain and mutant strains of the two transcriptional GL2 (pcoR-) and GLRFIA (rfiA-) transcript regulators and respective complemented strains GL2C and GLRFIAC (Licciardello *et al.,* 2007; 2009) against the fungus *Penicillium digitatum* eight day post inoculation.

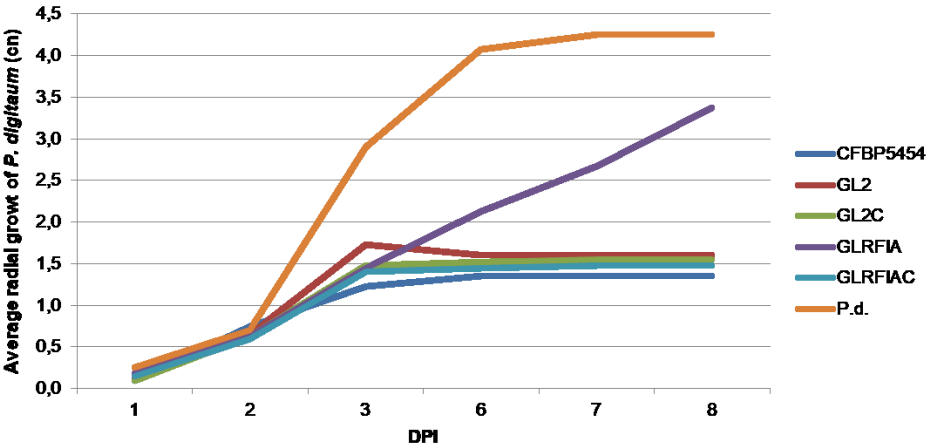

Figure 5. Time course inhibition of growth rate *in vitro* of *P. digitatum* induced by CFBP 5454, GL2, GLRFIA and relative complemented strains.

4.1.4. *In vivo* antagonistic activity against *P. digitatum*

The antagonistic activity of *P. corrugata* was evaluated in vivo on lemon fruits in relation to the pathogen *P. digitatum*, causal agent of green mould rot, based on the number of successful infections on the fruits and the average of disease index (DI). The infected areas due to the colonization of the pathogen were evaluated in comparison with the controls: fruits treated with the bacterial strains and inoculated with water (negative control), or treated with water and inoculated with the mushroom only (positive control).

The role of the two transcriptional regulators GL2 (*pcoR*-) and GLRFIA (*rfiA*-) on the antagonistic activity of *P. corrugata* was evaluated; for this, antagonistic activity of *P. corrugata* CFBP 5454 strain was compared with that of the mutant strains of two transcriptional regulator by analyzing the percentage of positive inoculations and the disease

index. In order to further test the role of the two transcriptional regulators, two bacterial strains were also included in the assay in which the mutations are complemented by insertion of carriers carrying a copy of the functional gene, respectively GL2C and GLRFIAC (Licciardello *et al.,* 2007; 2009).

Fruits treated with only *P. digitatum* three days after inoculation showed the first symptoms of the infection. These areas tended to widen and was observed the appearance of white mycelium and the formation of green spores in the center. Three days after inoculation, in fruits inoculated only with the bacterial strains (negative controls) no alterations were found 64% positive inoculations were recorded in fruits treated with only *P. digitatum* (positive control), 11% in fruits treated with CFBP 5454, 33% in those with GLRFIA, 58% with GL2, and in those with GL2C and GLRFIAC complemented strains 8% and 11% respectively (Fig.6). On the fifth day after inoculation, the percentage of positive inoculations increased reaching the 83% in the positive control, 19% in fruits treated with CFBP 5454, 50% and 69% in fruits treated with GLRFIA and GL2 respectively; 17% and 22% in the complemented strains GLRFIAC and GL2C respectively (Fig. 6). Five days after inoculation, a DI = 2 was observed in fruits treated with fungus only whereas in the other fruits it does not exceed DI = 1, except for those treated with GL2 which showed a mean DI index of 1.5 (Fig. 6).

ANOVA analysis and comparison of the averages with the Student-Newman-Kewls test ($P < 0.05$ and $P < 0.01$), both 3 and 5 days after inoculations showed that CFBP 5454, GLRFIA, GLRFIAC and GL2C significantly reduced the DI compared to the fruits treated with the fungus *P. digitatum*, while the GL2 treated fruits do not have a significant

difference from the positive control (Table 11). Considering the study of the *P. corrugata* action mechanisms and the results obtained from this test as a whole, it is apparent that the CFBP 5454 parental strain is very effective in reducing *P. digitatum* infections, and is distinguished appreciably as noted from the low incidence of positive infections, both from the low value of the disease index. The treatments performed with GL2 and GLRFIA mutant strains, in which PcoR and RfiA transcriptional regulators were inactivated, showed a reduced antifungal activity, significantly differing from parental strain treatments (CFBP 5454). Treatment with GL2 strain (*pcoR*-) does not differ significantly from inoculated treatments only with the fungus. In the treatments with GL2C and GLRFIAC supplemented strains, the activity of containment of *P. digitatum* infections was restored, in fact the mean of their Disease indexes (DI) did not differ significantly from that of CFBP 5454 parental strain.

4.1.5. Pathogenicity and HR

To examine the importance of AHL QS in tomato pith necrosis, the parental strain and *pcoR* (GL2) and *rfiA* (GLRFIA) knock-out mutant derivatives were inoculated into the stem of tomato plantlets in two different experiments. Using a high inoculum concentration of cells of *P. corrugata* CFBP 5454, the plants that were cross sectioned longitudinally, 1-week after inoculation, showed discoloration of the pith ranging from about 0.5 to 3 cm. The length of stem pith affected by discoloration of the plants inoculated with a GL2 mutant was significantly reduced ($P < 0.01$) compared to the length of the lesions caused by the parental strain, most of them being localised

around the inoculation point (average length 0.4 cm) (Fig. 7).

Plants inoculated with GLRFIA (*rfiA* mutant) lesions were significantly reduced in length compared with those caused by the parental strain and were only discolored (Fig. 7).

The effect of the inactivation of *pcoR* and *rfiA* on the ability to cause HR on tobacco was also explored. The tobacco leaf mesophyll was infiltrated with bacterial suspensions (10^8 CFU mL^{-1}) of the parental strain GL2 and GLRFIA mutants derivatives. By 24 h after inoculation the leaf panels infiltrated with the parental strain showed the collapse of the mesophyll, which turned necrotic within the following 24 h (Fig. 7). Leaf panels inoculated with the GL2 and GLRFIA mutants, as well as the water inoculated control, did not show any symptoms (Fig. 7).

Table 11. Statistical analysis of the means disease index recorded at 3 and 5 days post inoculation and of percentage of fungal growth inhibition.

	Disease index		
Treatment	3 d.p.i.	5 d.p.i.	**% of growth inhibition**
Penicillium digitatum	1,08 bB	1,75 cC	-
CFBP 5454 + P.d.	0,14 aA	0,28 aA	68,1 bB
GLRFIA + P.d.	0,5 aA	0,94 bAB	36,9 aA
GL2 + P.d.	0,97 bB	1,53 cBC	62,4 bB
GLRFIAC + P.d.	0,11 aA	0,25 aA	65,2 bB
GL2C+ P.d.	0,11 aA	0,28 aA	63,5 bB
Column values followed by equal letters do not differ significantly for $P < 0.05$ (lowercase letters) and $P < 0.01$ (uppercase letters) according to the Student-Newman-Kewls test.			

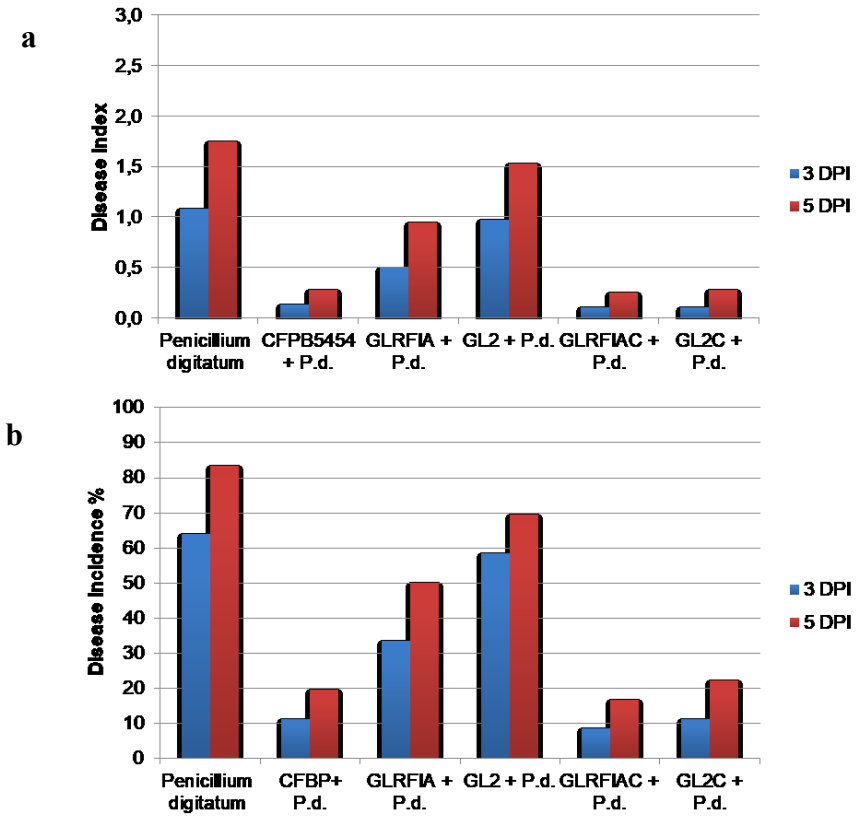

Figure 6. *In vivo* antagonistic activity of *P. corrugata* strains against *P. digitatum.* **a)** evaluation of disease index on lemon fruit treated with CFBP 5454, GL2, GLRFIA and respective complemented strains; **b)** evaluation of disease incidence on lemon fruit treated with CFBP 5454, GL2, GLRFIA and respective complemented strains.

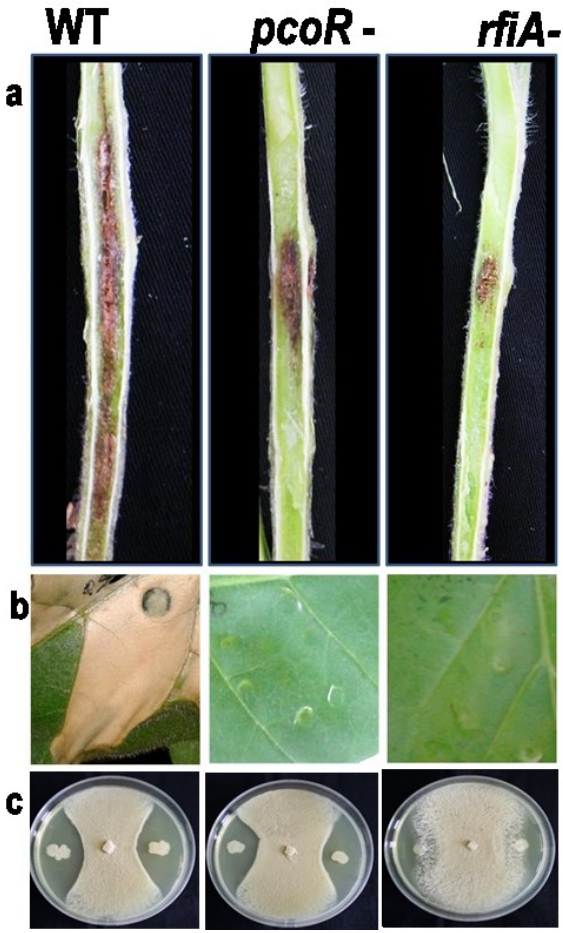

Figure 7. In planta inoculations and biological activity of *Pseudomonas corrugata* CFBP 5454 (WT), GL2 (*pcoR*- mutant) and GLRFIA (*rfiA*- mutant). **a)** Stem pith necrosis symptoms in prick-inoculated tomato plants (*L. esculentum*). **b)** Hypersensitivity reaction on tobacco leaves at 24 h post-inoculation with bacterial suspension. **c)** Evaluation of the *in vitro* antagonistic activity against the fungus *Penicillium digitatum* eight day post inoculation.

4.2. Updated genomic information on *Pseudomonas corrugata*

4.2.1. Phylogeny and genome neighbours

MLST showed that the *Pcor* strains cluster in distinct monophyletic clades within the larger *P. fluorescens* clade, clearly differentiated from *P. brassicacearum* subsp. *brassicacearum* NFM421T and *P. fluorescens* F113 and in the *P. fluorescens* lineage along with *P. mediterranea*, *P. kilonensis*, *P. thivervalensis,* and *P. brassicacearum* (the so called *P. corrugata* subgroup) (Trantas *et al.,* 2015 a). At the date of comparative genomics sequencing most of the genome of the subgroup were not available and the whole genome phylogenetic tree was performed on four *P. corrugata* strains and the closely related *P. mediterranea* (5 strains) and *P. brassicacearum* subsp. *brassicacearum* NFM421, *P. fluorescens* A506, *P. fluorescens* CHA0, *P. fluorescens* F113, *P. fluorescens* Pf0-1, *P. fluorescens* Pf-5, *P. fluorescens* SBW25, *P. syringae* pv. *syringae* B728a and *P. syringae* pv. *tomato* DC3000 as out-groups as already shown in the introduction.

Based on the genome sequence neighbours page of *P. corrugata* CFBP 5454 at NCBI is possible to see that more *P. corrugata* genome sequencing project are on going or finshed as well as those of other species in the *P. corrugata* taxonomic subgroup. Other than *P. corrugata* draft genome sequences published in the comparative genome study (namely CFBP 5454, CFBP5403, TEIC 1148, NCPPB 2445^{T}), three new projects are available at GeneBank repository. Among them there are the sequence of the strain DSM 7228^{T} which is the type strain designation at DSM and two strain complete genome sequences, BS 3649 and RM 1-1-4 (Table 12) (NCBI accession numbers in table 12).

Table 12. NCBI Genome Neighbor report for *P. corrugata* strain CFBP5454.

Species	Strain	Sccession # NCBI	Size (Mb)	Scaffolds	Symmetry identity (%)	Gapped identity %
Pseudomonas corrugata	NCPPB2445	NZ_LIGR00000000.1	6,08394	104	94.8402	99.5383
Pseudomonas corrugata	DSM 7228	NZ_LHVK00000000.1	6,12673	31	94.4560	99.5173
Pseudomonas corrugata	LMG 2172^{T}	NZ_LT629798.1	6,15084	-	94.3156	99.5329
Pseudomonas corrugata	RM 1-1-4	NZ_CP014262.1	6,12436	-	94.2533	99.5289
Pseudomonas corrugata	TEIC 1148	NZ_LIHH00000000.1	6,26658	431	92.4412	99.4941
Pseudomonas corrugata	CFBP 5403	NZ_LIGO00000000.1	6,16658	442	92.2707	99.4956
Pseudomonas mediterranea	TEIC 1022	NZ_LJWU00000000.1	6,23124	101	79.5455	92.9839
Pseudomonas mediterranea	CFBP 5403	NZ_LIGZ00000000.1	6,28154	150	79.1261	92.9590
Pseudomonas mediterranea	CFBP 5447	NZ_AUPB00000000.1	6,31969	32	79.0179	92.9638
Pseudomonas mediterranea	TEIC1105	NZ_LIGN00000000.1	6,29933	91	78.8891	92.9691
Pseudomonas mediterranea	DSM 16733	NZ_LT629790.1	6,34624	-	78.8889	92.9712
Pseudomonas mediterranea	CFBP5444	NZ_LIHG00000000.1	6,29886	89	78.8864	92.9699
Pseudomonas sp.	SHC52	NZ_CBLV000000000.1	6,31259	384	79.1104	93.4475

P. corrugata strain BS3649 is registered as LMG 2172^T (*P. corrugata* type strain at LMG collection).
Gapped identity percentage between *P. corrugata* strain CFBP5454 and other *P. corrugata* strains were of approximately the 99.5%. Whereas means value of the 92.9% were recorded with *P. mediterranea* strains. The bacterial strain *Pseudomonas* sp. SHC52 showed a gapped identity percentage of 93.4% towards *P. corrugata* CFBP5454. A cross check analysing the genome sequence neighbours of *Pseudomonas* sp. SHC52 showed that demonstrated that it shares a gapped identity percentage of about 92-93 with both species (data not shown).

4.2.2. Antismash prediction of secondary metabolite clusters in *P. corrugata* type strain

Several secondary metabolites gene cluster were found in the genomes of P. *corrugata* strain CFBP5454 and other three strains (CFBP5403, TEIC 1148, NCPPB 2445^T) analysed in a comparative genomics study (Trantas *et al.,* 2015 a). These includes a number of non-ribosomal peptide synthetases (NRPS) genes and clusters, and clusters putatively involved in biosynthesis of bacteriocin, polyketide synthase, siderophore and arylpolyene. Being *P. corrugata* CFBP5454 draft genome sequence split in 156 contigs most of the clusters comprising NRPS genes were divided and located in at least 10 different contigs making very difficult to assign them to a putative NRPS and product.
Since *P. corrugata* strains CFBP 5454 and LMG 2172^T have a genome ANI of 99.53% we therefore used the annotated sequence of the latter strain for the in silico

mining of secondary metabolite clusters. The genome of *P. corrugata* strain LMG 2172^T was subjected to an automated search using the "antibiotic and secondary metabolite analysis shell" antiSMASH 3.0 (Blin *et al.,* 2013) for the automated identification of secondary metabolite biosynthesis clusters. The analysis applying default setting allowed the identification of seven secondary metabolite clusters (Table 13; Appendix 1) accounting for approximately the 7.2 % of the genome: two non-ribosomal-peptide synthetase (NRPS) clusters one of which associated to an homoserine-lactone synthase gene (HSL-NRPS); siderophore; bacteriocin; polyketide synthetase (PKS) and one generally classified as "Other".

Cluster 1 recorded as HSL-NRPS cluster is the largest one accounting for approximately the 3.4 % of the genome (Fig. 8; Appendix 2). This large cluster includes 6 NRPS genes. Based on NRPS predicted aminoacid lipopeptides and on the recently described similar gene clusters in *Pseudomonas* sp. SH-C52 (Van der Vort *et al.,* 2015) and in *P. fluorescens* In5 (Hanessey *et al.,* 2017), in which the production of CLPs similar to those produced by *P. corrugata* were described. The NRPS were putatively attributed to three very closed biosynthetic clusters for the synthesis of the CLPs of the class of peptins constituted by a 22 amino-acidlipopeptide namely corpeptins and the nonapeptide cormicyn similarly to nunapeptins and thanapeptins and nunamycin and thanamycin in *Pseudomonas fluorescens* In5 (Michelsen *et al.,* 2015; Hanessey *et al.,* 2017) and *Pseudomonas* sp. SH-C52 (Mendes *et al.,* 2011; Van der Vort *et al.,* 2015) respectively. In close proximity to the cor/thana/nunmycin cluster a third NRPS constituted by two adenilation domains was identified in P. *corrugata* strain LMG 2172^T as well as in the other P. *corrugata* strains (data

not shown), in In5 and SH-C52 (Van der Voort *et al.,* 2015; Hennessy *et al.,* 2017). This NRPS code in SH-C52 for the synthesis of a dipeptide designed brabantamide (Schmidt *et al.,* 2014) which cluster is also present in In5 (Hennessy *et al.,* 2017). Closed to these NRPS cluster many genes coding for proteins putatively involved in transport and regulation. In particular the ending part of the last corpeptin NRPS gene previously described form an operon designated crpCDE with genes coding for an ABC efflux system, mutant in this gene were not able anymore to produce corpeptins (Strano *et al.,* 2015), the pcoABC operon coding for an RND efflux system (Licciardello *et al.,* 2009). This cluster also includes genes for the PcoI/PcoR AHL-QS system, the transcriptional regulator gene RfiA (Licciardello *et al.,* 2007; 2009). These sequences were already present in the GenBank sequence KF192265. The analysis of NRPS clusters in the other strains supported the presence of the same cormycin and corpeptin biosynthesis, secretion and regulatory apparatus for all strains (data not shown).

The other cluster of secondary metabolites were listed below. The PKS cluster was predicted to encode insecticidal toxin similar to the entolysin cluster. The bacteriocin gene clusters could be identified with similarity to those found in other genomes of the *P. fluorescens* clade. Of the two additional NRPS gene clusters one is presumably involved in the (orni)corrugatin biosynthesis a secondary siderophores described by Matthijs *et al.,* (2008) and the second one shows similarity to *mgoA*-like gene clusters, encoding only one adenylation domain. MgoA or MgoA-regulated compound(s) were proposed to regulate the expression of pathogenicity factors in *P. entomophila* and *P. syringae* (Vallet-Gely *et al.,* 2010; Carrion *et al.,* 2014), and were also found in *Pseudomonas* sp. SHC52 (Van der Voort

et al., 2015). The siderophore gene cluster for achromobactin, described for *P. syringae* strains (Berti and Thomas, 2009; Owen and Ackerley, 2011) and for two *P. chlororaphis* strains (Loper *et al.,* 2012) was detected although it seems that lack of the transporter genes described in the the latter species as described already by Trantas *et al.,* (2015 a). In addition, *P. corrugata* strain LMG 2172^T, as well as the other *P. corrugata* strains, has the gene cluster for hydrogen cyanide production, that it has been demonstrated to have a role in *P. corrugata* CFBP5454 biocontrol (Strano *et al.,* 2017).

4.2.3. PcoR and RfiA homologs in *Pseudomonas* sp.

Trantas *et al.* (2015 a) found in the genomes of *P. corrugata* strains CFBP5403, TEIC 1148, NCPPB 2445^T as well as in five strains of the closely related *P. mediterranea* that all strains have genes of only one canonical paired LuxI/R system (PcoI/R in *P. corrugate* and PmeI/R in *P. mediterranea*) and have the *rfiA* gene coding for a transcriptional regulator downstream of, and in the same orientation as, *luxI* gene as previously described (Licciardello *et al.,* 2007; 2009; 2012).

Pseudomonas CLPs biosynthesis cluster are flanked by genes coding for LuxR transcriptional regulators (reviewed in Raaijmakers *et al.,* 2010) but up to recently the presence of a LuxR regulator directly linked to an AHL-QS system by mean of gene cotranscription with acylhomoserine lactone synthase gene was described only for *P. corrugata* and *P. mediterranea* (Licciardello *et al.,* 2012). Since numerous genomes have been obtained since their first description a BLASTX analysis using the sequence of *pcoR*

and *rfiA* was conducted on the genome of the *Pseudomonas* species of the *P. corrugata* subgroup.
The QS gene topology was conserved in both *P. corrugata* and *P. mediterranea* strains as well as *Pseudomonas* sp. SCH52, with the *luxI/luxR* genes oriented in the same direction and separated by a divergently oriented ORF, which codes for a putative homoserine/threonine efflux protein.
P. corrugata CFBP 5454 PcoI, PcoR and RfiA showed protein homologies of the 100% with the corresponding proteins in *P. corrugata* strain LMG 2172^{T}, of 85% 95% and 94% with strain *Pseudomonas* sp. SHC52, and of 84%, 95% and 92% with *P. mediterranea* strain DSM16733^{T}, respectively (Table 14).

Table 13. Results of the analysis with Antismash on the genome of *P. corrugata* strain LMG 2171^T.

Cluster	Type	From	To	Most similar known cluster	MIBiG BGC-ID	% on total genome
Cluster 1	Hserlactone-Nrps	1502712	1711350	Syringomycin_biosynthetic_gene_cluster (100% of genes show similarity)	BGC0000437_c1	3.39
Cluster 2	Siderophore	2160746	2179665	-	-	0.31
Cluster 3	T1pks	2327254	2375719	Entolysin_biosynthetic_gene_cluster (17% of genes show similarity)	BGC0000344_c1	0.79
Cluster 4	Nrps	2573053	2647389	Cupriachelin_biosynthetic_gene_cluster (11% of genes show similarity)	BGC0000330_c1	1.21
Cluster 5	Bacteriocin	3700046	3710933	-	-	0.18
Cluster 6	Arylpolyene	4606895	4650506	APE_Vf_biosynthetic_gene_cluster (45% of genes show similarity)	BGC0000837_c1	0.71
Cluster 7	Other	4891179	4934715	Mangotoxin_biosynthetic_gene_cluster (71% of genes show similarity)	BGC0000387_c1	0.71
					Total	7.20

Table 14. Percent nucleotide and amino acid identities of *pcoI* and *pcoR* *P. corrugata* CFBP5454 QS system and *rfiA* transcriptional regulator within *P. corrugata* subgroup after pairwise alignment by blastn and blastx.

Genome Name / Sample Name	**blastn**	**blastx**	**blastn**	**blastx**	**blastn**	**blastx**
	pcoI	*pcoI*	*pcoR*	*pcoR*	*rfiA*	*rfiA*
P. corrugata LMG 2172	772/774 99%	257/257 100%	728/729 99%	242/242 100%	677/678 99%	225/225 100%
P. sp. SHC52	609/705 86%	96/113 85%	662/727 91%	231/242 95%	620/677 92%	212/225 94%
P. mediterranea DSM 16733	661/778 85%	217/257 84%	654/729 90%	230/242 95%	621/680 91%	208/225 92%
P. brassicacearum LMG 21623	-	-	-	-	-	-
P. kilonensis DSM 13647	-	-	-	-	-	-
P. thivervalensis DSM 13194	-	-	-	-	-	-
P. frederiksbergensis LMG 19851	-	-	-	-	-	-

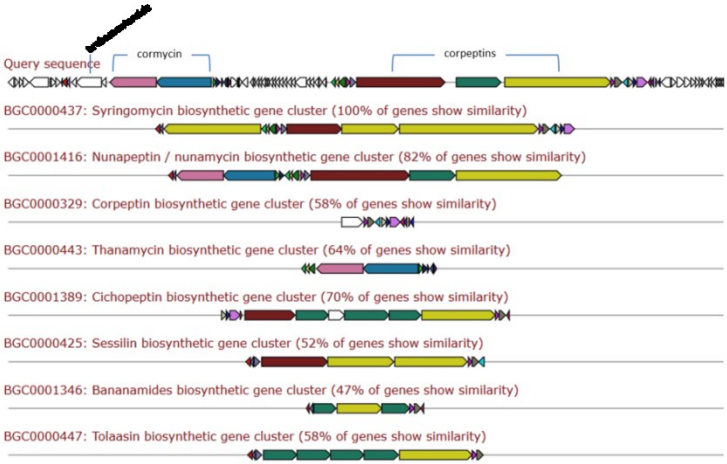

Figure 8. Antismash analysis of strain LMG 2171^{T}: Cluster 1- HSL-NRPS cluster homology analysis.

4.2.3. Alginate gene cluster

In a previous study, Licciardello *et al.,* (2017) described for the first time the presence of the alginate biosynthetic and regulatory cluster in *P. mediterranea* CFBP5447 demonstrating also its ability to produce high quantity of exopolysaccarides. Because this cluster has been detected also within the *Pseudomonas* sp. SH-C52 genome, we examined also the genomes of *P. corrugata* CFBP5454 as well as of other *P. corrugata* strains already sequenced for the presence of this cluster. Although Fett *et al.* (1992) described that *P. corrugata* strain 388 contained genes with homology with alginate biosynthetic and regulatory genes cloned from the alginate producing bacterium *P. aeruginosa*, the gene cluster wasn't highlighted in the *P. corrugata* genome studies (Licciardello *et al.,* 2014; Trantas *et al.,* 2015 a).

We therefore performed *P. corrugata* CFBP5454 genome mining analysis which revealed that, similarly to other *Pseudomonas* rRNA homology group I, the core alginate biosynthesis and regulatory genes are widely distributed into three clusters namely, structural/biosynthetic, regulatory and genetic switching genes (Table 15) (Schurr *et al.,* 1993; Shankar *et al.,* 1995, Fiahlo *et al.,* 1990, Fett *et al.,* 1992) RIF). According to *P. aeruginosa* studies, the core genes involved in alginate biosynthesis are contained within a single 12-gene operon initially described by Chitnis and Ohman (1993), including *algD*, *alg8*, *alg44*, *algK*, *algE*, *algG*, *algX*, *algL*, *algI*, *algJ*, *algF*, *algA* and *algC* (Fig. 9). The genes are under the tight control of a promoter upstream of algD (Schurr *et al.,* 1993; Shankar *et al.,* 1995), although there is some evidence to suggest that alternative internal promoters exist within the operon (Lloret *et al.,* 1996; Paletta and Ohman, 2012). The gene algC is not located within the operon and is also involved in rhamnolipid and lipopolysaccharide biosynthesis (Olvera *et al.,* 1999) These genes are clustered in the *P. corrugata* CFBP5454 genome within an 18 kb region located in the contig86 except *algC* which is located within the contig46. Additional copies of *algE* (*algE2_1, algE_3, algE5*) are dispersed in other contigs (Table 15, Fig. 9)

The same cluster was evidenced in all *P. corrugata* genomes as well as in the other species of the P. *corrugata* clade within the *P. fluorescens* group. Nucleotide identity of *algD*, *algG* and *algI* after pairwise alignment by blastn ranged from 99% to 89% for *Pseudomonas corrugata* LMG 2172 and *Pseudomonas frederiksbergensis* LMG 1985, respectively (Table 16). Blastx homology analysis showed amino acid identities ranging from 100% to 94% for AlgD, 100%-88% for AlgG and 100%-93% for AlgI for

Pseudomonas corrugata LMG 2172 and *Pseudomonas frederiksbergensis* LMG 1985, respectively (Table 16). In addition to these 13 core genes involved in alginate biosynthesis, many more have been identified and are summarized in Table 15. *algI, algJ*, and *algF*, suggested to encode a family of proteins involved in the esterification of surface or extracellular polysaccharides in a variety of bacteria, are located in a distant locus within the contig 3, because of a lateral gene transfer according to Frankling *et al.* (2004).

The cluster of alginate regulatory genes in *P. corrugata* is composed of five genes and includes *algR*, *algQ, algP, algB* and *kinB*. *algR*, *algQ* and *algP* are localized in the same genomic region (contig86) but not immediately adjacent, similarly to *P. aeruginosa*, although *P. corrugata* lacks the algZ regulatory gene (Deretic *et al.,* 1989). *algB* and *kinB* are located in contig45 in the same transcriptional unit and coded for a two-component system where AlgB is the response regulator and KinB the sensor hystidine kinase. The alginate switching genes which mediate the conversion to constitutive Alg+ phenotype, consisted of four genes, *algU, mucA, mucB* and *mucD* located in the contig144 of *P. corrugata* clustered together in the same transcriptional unit.

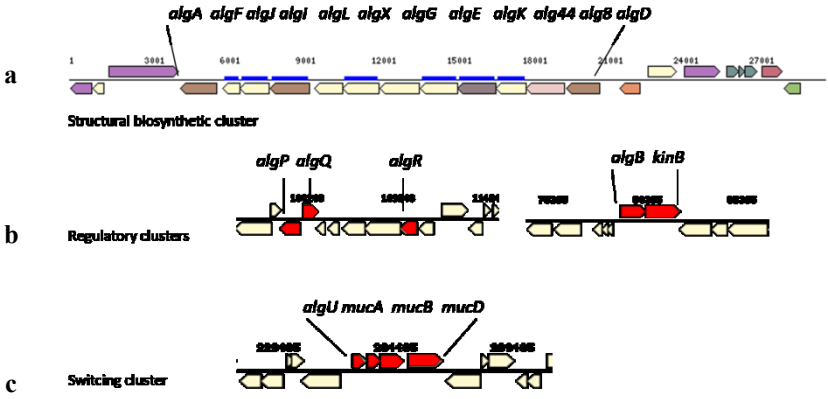

Figure 9. Organization of the clusters responsible of alginate bacterial production. **a)** Structural/biosynthetic cluster composed of 12 genes and located in the contig 38 of *P. corrugata* CFBP5454 draft genome; **b)** Regulatory clusters located in contig 86 and contig 45; **c)** Switcing cluster located in contig 144.

Table 15. Genes involved in alginate biosynthesis annotated within the *P. corrugata* CFBP5454 genome.

Gene cluster	Gene	Contig	locus_tag	Gene product
Structural/ biosynthetic	*algD*	contig38	PROKKA_02169	GDP-mannose 6-dehydrogenase
	alg8	contig38	PROKKA_02168	Glycosyltransferase
	alg44	contig38	PROKKA_02169	Alginate biosynthesisprotein
	algK	contig38	PROKKA_02170	Alginate biosynthesisprotein
	algE	contig38	PROKKA_02171	Alginate production protein
	algG	contig38	PROKKA_02172	Poly(beta-D-mannuronate) C5 epimerase
	algX	contig38	PROKKA_02173	Alginate biosynthesisproteinAlgX
	algL	contig38	PROKKA_02174	Alginate lyase
	algI_1	contig38	PROKKA_02175	putative alginate O-acetylase
	algJ_1	contig38	PROKKA_02176	putative alginate O-acetylase
	algF	contig38	PROKKA_02177	Alginate biosynthesis protein
	algA	contig38	PROKKA_02178	Alginate biosynthesis protein
	algC	contig86	PROKKA_04584	Phosphomannomutase/phosphoglucomutase
	algE5	contig47	PROKKA_01144	Poly(beta-D-mannuronate) C5 epimerase
	algE2_1	contig32	PROKKA_02846	Poly(beta-D-mannuronate) C5 epimerase 2
	A lgF	contig3	PROKKA_03653	Alginate O-acetyltransferaseAlgF
	algI_2	contig3	PROKKA_03652	putative alginate O-acetylase
	algJ_2	contig3	PROKKA_03651	putative alginate O-acetylase
	algE3	contig25	PROKKA_01240	Poly(beta-D-mannuronate) C5 epimerase 3
	algE2_2	contig46	PROKKA_02846	Poly(beta-D-mannuronate) C5 epimerase 2
Regulatory	*algR*	contig86	PROKKA_04531	Positive alginate biosynthesis regulatory protein
	algQ	contig86	PROKKA_04526	Transcriptional regulatory protein
	algP	contig86	PROKKA_04524	Transcriptional regulatory protein
	algB	contig45	PROKKA_01267	Alginate biosynthesis transcriptional regulatory protein
	kinB	contig45	PROKKA_01266	Alginate biosynthesis sensor protein kinB
Genotypic switching	*algU*	contig144	PROKKA_03270	Sigma factor AlgU regulatory protein
	mucA	contig144	PROKKA_03269	Sigma factor AlgU negative regulatory protein
	mucB	contig144	PROKKA_03268	putative periplasmic serine endoproteaseDegP-like
	mucD	contig144	PROKKA_03267	putative periplasmic serine endoproteaseDegP-like

Table 16. Percent nucleotide and aminoacid identities of *algD, algG* and *algI P. corrugata* CFBP5454 within the *P. corrugata* subgroup after pairwise alignment by blastn and blastx.

Genome Name / Sample Name	IMG Genome ID	blastn	blastx	blastn	Blastx	Blastn	blastx
		algD	***algD***	***algG***	***algG***	***algI_1***	***algI_1***
P. corrugata LMG 2172	2639763195	1313/1314 99%	438/438 100%	1606/1608 99%	529/529 100%	1562/1563 99%	521/521 100%
Pseudomonas sp. SHC52	2627854252	1250/1314 95%	432/438 99%	1551/1608 96%	517/529 98%	1510/1563 97%	520/521 99%
P. mediterranea DSM 16733	2675902957	1262/1314 96%	435/438 99%	1567/1608 97%	521/529 98%	1513/1564 97%	517/521 99%
P.brassicacearum LMG 21623	2639762602	1228/1314 93%	430/438 98%	1503/1609 93%	507/529 96%	1476/1564 94%	508/521 98%
P. kilonensis DSM 13647	2713897225	1228/1314 93%	428/438 98%	1491/1612 92%	496/530 94%	1453/1563 93%	501/521 96%
P. thivervalensis DSM 13194	2675903515	1221/1314 93%	427/438 97%	1507/1609 94%	501/529 95%	1469/1564 94%	504/521 97%
P. frederiksbergensis LMG 19851	2636416079	1170/1315 89%	412/438 94%	1292/1456 89%	443/504 88%	1387/1566 89%	482/521 93%

4.2.4. *P. corrugata* genome

According to the results of comparative genomics of *P. corrugata* and *P. mediterranea* (Trantas *et al.,* 2015 a), the genome mining analysis of the *Pseudomonas* sp. SHC52 on strains/species of the *P. corrugata* subgroup and the new data mined on *P. corrugata* type strain genome a pictorial representation of secondary metabolites and secretion systems was depicted in Figure 10. Among the main characteristic of the species other than the antimicrobial molecules it is to be highlighted the absence of a T3SS as it was also reported in *P. mediterranea* (Trantas *et al.,* 2015 a) and in *Pseudomonas* SHC52 (Van der Voort *et al.,* 2015).

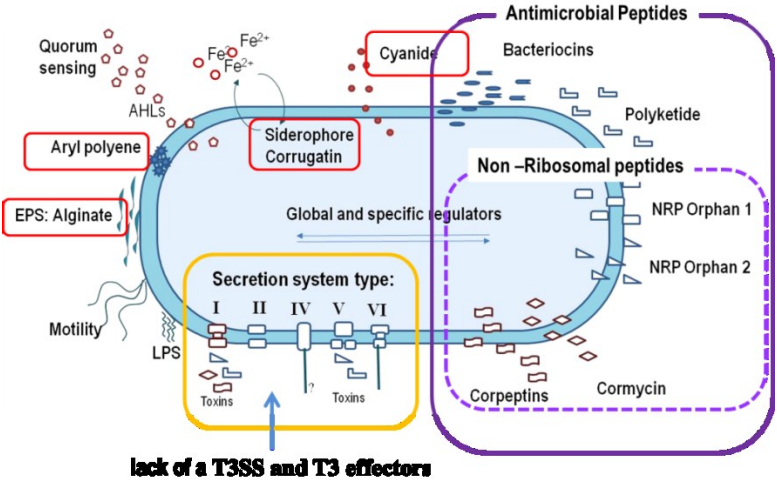

Figure 10. Pictorial representation of secondary metabolites and secretion systems identified in four *P. corrugata* genomes based on genome-mining. The analysis revealed the absence of type III secretion system and known type III effector-encoding genes a circumstance unique among the plant pathogenic pseudomonads. The presence of gene clusters for biosynthesis of siderophores, polyketides, Non-Ribosomal Peptides, cyanide (HCN), alginate was evidenced. A highly conserved quorum sensing system was also detected (Licciardello *et al.,* 2014; Trantas *et al.,* 2015 a).

4.3. Differential expression analysis of the transcriptome of *P. corrugata* CFBP5454 *pcoR* and *rfiA* mutants

4.3.1. RNA-seq analysis of *pcoR*- and *rfiA*- mutants in *P. corrugata* CFBP5454

To investigate on the regulatory functions of PcoR and RfiA transcriptional regulators, expression profiles from RNA-seq data were analysed. PcoR and RfiA are regulators belonging to the LuxR family protein, although to different subfamilies, that control large number of genes also in other *Pseudomonas* spp. (Vaughn *et al.,* 2016, Papenfort and Bassler, 2016). However, this is a singular case in which the *rfiA* transcription is directly controlled by QS system by co-transcription with *pcoI*.

We compared transcriptomes of wild-type *P. corrugata* CFBP5454 with those of the mutant strains GL2 (*pcoR*-) and GLRFIA (*rfiA*-) (Licciardello *et al.,* 2007; 2009) grown to early stationary phase cultures of three biological replicates growing on IMM at static conditions inducing CLPs production after 40 hrs of incubation. Because in previous studies, the role of these regulators in CLPs production has been demonstrated, we decided to analyse the transcriptome in conditions favoring these secondary metabolites (Licciardello *et al.,* 2007). Single-stranded cDNA libraries were sequenced and mapped against *P. corrugata* CFBP5454 reference genome (ATKI01000000). Genes with increased expression in the wild-type strain as compared to the mutant strains were considered to be positively regulated, either directly or indirectly, by PcoR or RfiA. Otherwise, genes with enhanced expression in mutants were considered negatively regulated by transcriptional regulators in the wild-type strain.

Differential expression analysis between the Wt strain CFBP5454 either the GL2 (*pcoR-)* or GLRFIA (*rfiA-)* derivative mutant strains showed that, with a false discovery rate (FDR) correction of 5%, 152 genes (46 increased and 106 decreased) were identified as significantly different in the GL2 mutant, while 130 genes (52 increased and 78 decreased) were identified as differentially expressed in the GLRFIA mutant. Among them, 92 genes, which represent the 3% of the annotated genes in the CFBP 5454 draft genome, were both PcoR- and RfiA-regulated at the transcriptional level whereas 60 and 38 were independently regulated either by PcoR or RfiA, respectively (Fig. 11, 12). In order to assemble a catalogue of functions strongly linked to the role of these transcriptional regulators, differentially expressed genes were grouped based on GO by using Gene Ontology Consortium (http://geneontology.org/). Genes were grouped into 14 functional categories on the basis of PseudoCAP and were plotted with respect to down-regulation and up-regulation (Fig. 13, 14). The largest group consisted of enzymes associated to transport systems, 34 of which were differentially expressed in the GL2 mutant and 27 in the GLRFIA mutant compared with the expression on parental strain. They were followed at a distance by genes involved in redox and oxidative stresses, most of them are down-regulated in both GL2 (17) and GLRFIA (11) mutants. Genes predicted to be related with alginic acid biosynthesis process (12 genes) and secondary metabolites production (11 genes) are well represented among the downregulated genes in both mutants revealing a predominant positive control of both PcoR and RfiA in the biosynthesis of these molecules. The category of transcriptional regulator genes account a relevant number of transcripts affected by *pcoR* and *rfiA* mutations, among up-

(8) and down- (11) regulated genes showing a wide control through a cascade of other regulators. Other gene categories affected are involved in carbohydrate metabolic process, fatty acid, aminoacid and purine and pyrimidine metabolisms.
Supplementary files contains a thorough analysis of the transcripts and their cognate predicted functions that were found associated to the role of PcoR and RfiA (Appendixes III-VII).

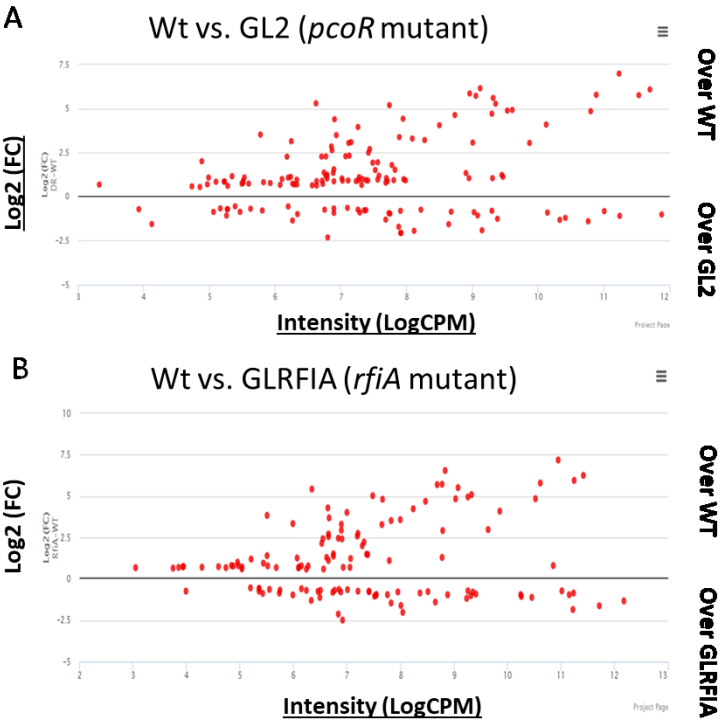

Figure 11. Graphical representation of the relationship between intensity (LogCPM) and difference (Log2FC) of genes between *P. corrugata* CFBP5454 (WT) vs GL2 (A) and GLRFIA (B) derivatives mutants built with DEPICTViz, Differential Expression and Protein Interactions Visualization tool (http://jau.facom.ufms.br/Pcor/). In the plot red points represent genes that were classified as differentially expressed (FDR <0.05). Points along the x-axis represents the average intensity of gene expression between conditions. The points above '0' on the y-axis represent genes that are more expressed in WT condition than in GL2 (A) or GLRFIA (B). Points below '0' on the y-axis represent genes that are more expressed in GL2 (A) or GLRFIA (B) condition than in WT.

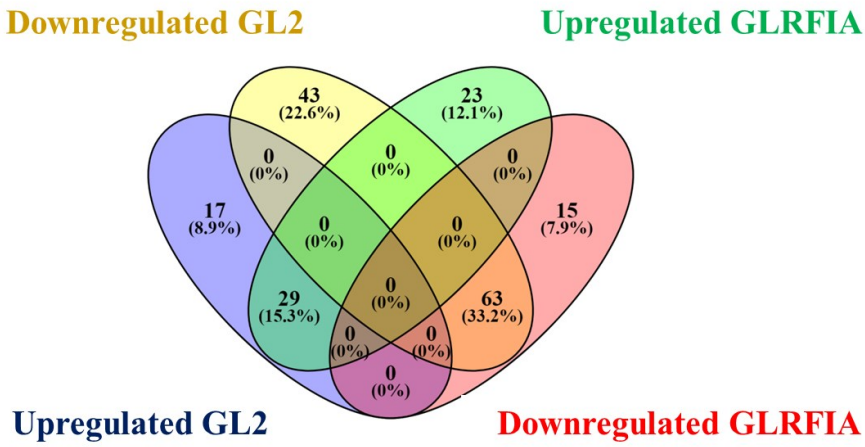

Figure 12. Venn diagram showing the relationship between the PcoR and RfiA regulons as determined by RNAseq analysis in *P. corrugata* wild-type strain in comparison to GL2 and GLRFIA mutants strains ($P \leq 0.05$). The diagram shows the degree to which the regulons overlap between PcoR and RfiA based on the model of their opposing effects on the expression of many genes. In the diagram, built with VENNY 2.1 software (*Oliveros, J.C. 2007-2015),* is indicated the number of genes upregulated or downregulated in wild type in comparisons to the mutants.

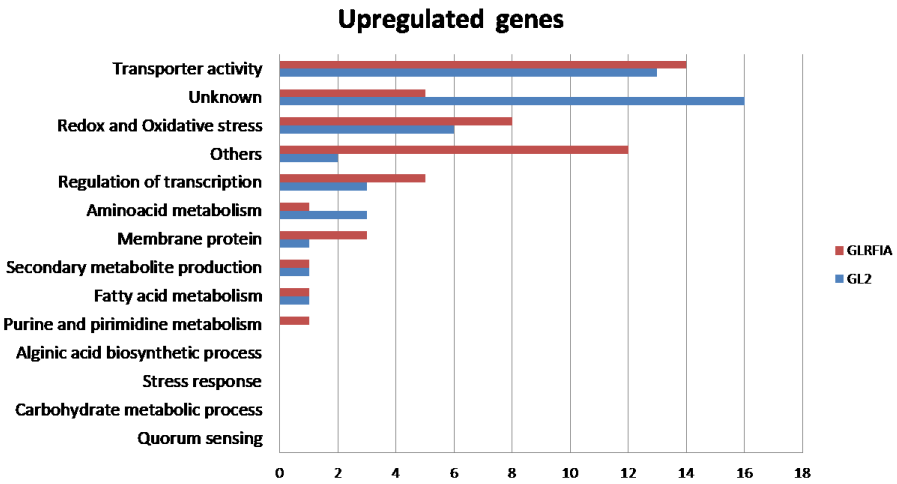

Figure 13. Number and functional classification of genes up-regulated in *P. corrugata* GL2 and GLRFIA derivative mutants in comparison to the WT strain CFBP5454. The plot indicates the type of physiological role(s) and the total number of genes with increased expression within that category.

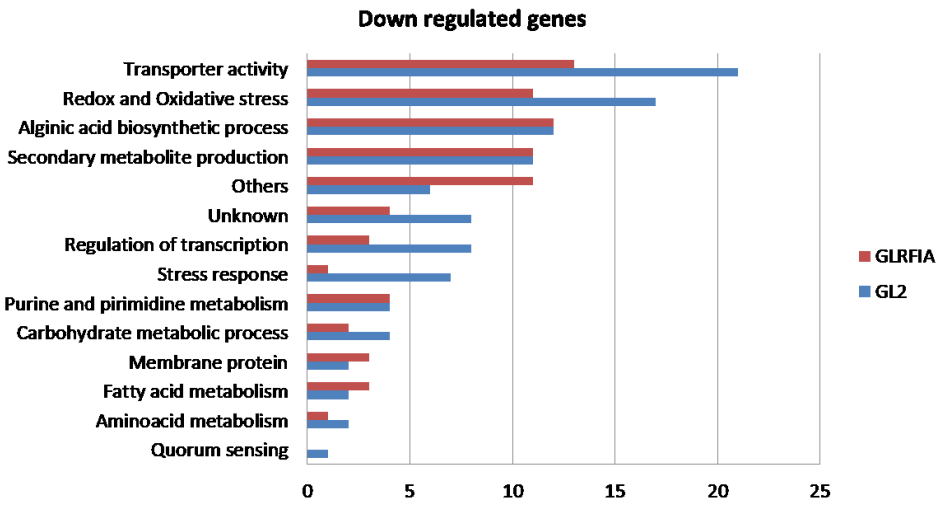

Figure 14. Number and functional classification of genes down-regulated in *P. corrugata* GL2 and GLRFIA derivative mutants in comparison to the WT strain CFBP5454. The plot indicates the type of physiological role(s) and the total number of genes with decreased expression within that category.

4.3.2. Cyclic Lipopeptide and Alginate biosynthetic clusters are part of the PcoR-RfiA regulon

According to RNAseq analysis, the 3% of total annotated genes in the genome of *P. corrugata* CFBP5454, resulted to be affected by both *pcoR* and *rfiA* inactivation. A total of 184 transcripts corresponding to the was down regulated in both mutants demonstrating a strong activator role of these transcriptional regulators which seems to work as a unique regulon during growth of *P. corrugata* in IMM minimal medium.

As already described, *P. corrugata* produces cormycin and corpeptins (Emanuele *et al.,* 1998; Strano *et al.,* 2015). Also our strain is able to produce both molecules as demonstrated by Licciardello *et al.,* (2012). During studies on the role of QS system, it was evidenced that *pcor* and *rfiA* mutants are not still able to produce cormycin and corpeptins (Licciardello *et al.,* 2012). Blast analysis allowed to ascertain that among genes differentially expressed in RNAseq analysis, 20 clusters involved in CLPs production are regulated by PcoR and RFIA (Table 17, Fig. 15).

Specifically, the whole biosynthetic cluster of corpeptin composed of three non ribosomal peptide synthetases (NRPS), two ABC transporters genes, and one RND transporter are under the transcriptional control of both regulators. This is in accordance with previous data shown by Licciardello *et al.,* (2009) and Strano *et al.,* (2015, 2016) which demonstrate, by mutational analysis, that *pcoABC* and *crpCDE* are involved in corpeptin biosynthesis and secretion. In fact, the corpeptin NRBP gene (*ppsE* correspondent to *crpC*) was significantly upregulated in the strain in comparison to GL2 and GLRFIA mutants by 5.78 and 5.79 fold, respectively (Appendixes III-IV). Similarly genes involved in corpeptin transport, such as *macA* and

macB2 (corresponding to *crpDE*) and those of RND transporter system namely *pcoABC* were significantly upregulated in the wild-type in accordance to our previous results which demonstrated the active role of PcoR and RfiA in regulating CLPs export out from the cell. Although the *pcoR* and *rfiA* mutants failed to produce cormycin, none of the NRPS genes involved in this pathways was evidenced among the differentially expressed genes. However, we found two genes coding for ABC transporter systems already described in others *Pseudomonas* spp. coding for, for example, the siringomycin or thanamycin transporters and for a gene annotated as *syrD* whose homologue in *P. syringae* has been shown to play a role in the secretion of syringomycin and syringopeptin (Quigley *et al.,* 1993). These genes are overexpressed in the wild-type CFBP5454 strain in comparison to GL2 and GLRFIA mutants (5.28 and 5.51 fold, respectively). We have also identified among those differentially expressed, the *yeaM* gene coding for a transcriptional regulator, flanking the putative cormycin NRPS genes, overexpressed 3.05 and 2.93 fold in the wild-type in comparison to GL2 and GLRFIA, respectively. In the downstream region of genes responsible of cormycin biosynthesis we identified a cluster detected by Antismash tool, as similar to brabantamide synthase gene described in *Pseudomonas* sp. SHC52 (Van der Voort *et al.,* 2015). Four genes of this cluster have been found to be differentially expressed in *pcoR* and *rfiA* mutants. Although this metabolite has not been described yet in *P. corrugata* CFBP5454, it is suggested that previous experimental conditions not allowed to detected it (Emanuele *et al.,* 1998; Scaloni *et al.,* 2004).

By the fact the results are in agreement with phenotypic characterization of the mutants *P. corrugata* CFBP 5454 as well as its cell-free culture filtrates produced in IMM minimal medium showed antimicrobial activity against the two CLP bioindicators, the yeast *Rhodotorula pilimanae* ATCC26423 and the Gram-positive bacterium *Bacillus megaterium* ITM100 as previously described. Cell-free culture filtrate antagonistic activity of both Lux regulators mutants was abolished (Figure 16). Notably GL2 (*pcoR* mutant) and GLRFIA (*rfiaA* mutant) strains when spotted on NDA agar plates were still able to inhibit the growth of the bacterium *B. megaterium* ITM100.

Antagonistic activity against the fungus *P. digitatum* evidenced that the *pcoR* mutant is still able to inhibit the growth of *P. digitatum* to the level of the parent strain and that the *rfiA* mutant have a reduced activity and the fungus over the time overgrowth the bacterial spot. Thus more metabolites that are not regulated by the PcorR-RfiA regulon are involved or at least no transcripts for these gene were obtained in our experimental conditions.

In addition as showed before tomato plants inoculated with the mutant strains showed a drastically reduced virulence and were not able anymore to induce HR on *Nicotiana tabacum* and *N. benthamiana* leaf mesophyll.

In addition, for the first time, here we report that all the gene cluster involved in alginic acid biosynthesis is affected by PcoR and RfiA.

RNAseq analysis revealed that twelve genes belonging to the structural/biosynthetic cluster of alginate (Fig. 9) were upregulated in wild-type strain CFBP 5454 in comparison to both GL2 and GLRFIA mutant strains with LogFC ranging from 4.09 (*algD*) to 1.93 (*algG*) (Appendixes III-IV). No

other gene located in alginate regulation and switching operons was altered in these mutants.
Total EPSs were isolated after isopropanol precipitation from the supernatant of the GL2 and GLRFIA mutant strains growing in two different substrates, IMM and NB, compared to the *P. corrugata* parent strain with the aim to validate transcriptional results. In IMM minimal medium the yield of lyophilized EPSs produced by the Wt strain was 82 mg/100 ml whereas in GL2 and GLRFIA mutant strains was 6 mg/100 ml and 10 mg/100 ml, respectively. These data showed an about 10-fold reduction of EPSs yield in both mutants confirming RNA-seq results and the active role of PcoR and RfiA in regulating alginate production (Fig. 17).

Table 17. *Pseudomonas corrugata* CFBP5454 Locus tag of genes of the secondary metabolite of cluster 1 identified by Antismash analysis of strain LMG 2172 and regulated by PcoR-RfiA regulon.

PCFBP5454 Locus tag	LMG2172 Locus tag	Putative gene	Description	Function	LogFC GL2	LogFC GLRFIA
K659_RS0103735	BLU14_RS07135	arpC	Antibiotic efflux pump outer membrane protein ArpC	membrane protein	3.07	2.92
K659_RS0103725	BLU14_RS07145	hpxO	FAD-dependent urate hydroxylase	Purine and pirimidine	4.63	4.69
K659_RS0103720	BLU14_RS07150	dhbF_1	Dimodular nonribosomal peptide synthase	Secondary metabolite production	4.85	4.84
K659_RS0103715	BLU14_RS07155	ATG26	Sterol 3-beta-glucosyltransferase	transporter activity	5.72	5.72
K659_RS0121340	BLU14_RS07170	Hif1 an	Hypoxia-inducible factor 1-alpha inhibitor	Redox and Oxidative stress	3.95	4.01
K659_RS0121335	BLU14_RS07175	mefA	Macrolide efflux protein A	transporter activity	3.49	3.68
K659_RS0121325	BLU14_RS07185	yeaM_3	putative HTH-type transcriptional regulator YeaM	regulation of transcription	3.05	2.93
K659_RS0123940	BLU14_RS07330	pcaD_2	3-oxoadipate enol-lactonase 1	Secondary metabolite production	4.89	4.94
K659_RS0123930	BLU14_RS07340	dhbF_3	Dimodular nonribosomal peptide synthase	Secondary metabolite production	5.19	7.18
K659_RS0123920	BLU14_RS07345	At3g21360_3	Clavaminate synthase-like protein	Redox and Oxidative stress	6.15	6.55
K659_RS0123925	BLU14_RS07350	syrD_2	ATP-binding protein SyrD	Secondary metabolite	5.28	5.51

PCFBP5454 Locus tag	LMG2172 Locus tag	Putative gene	Description	Function	LogFC GL2	LogFC GLRFIA
				production		
K659_RS01000000128675	BLU14_RS07355	tycB	Tyrocidine synthase 2	Secondary metabolite production	5.30	5.42
K659_RS01000000128480	BLU14_RS07360	dhbF_4	Dimodular nonribosomal peptide synthase	Secondary metabolite production	5.19	5.02
K659_RS01000000128500	BLU14_RS07365	grsB_2	Gramicidin S synthase 2	Secondary metabolite production	6.08	6.25
K659_RS0121920	BLU14_RS07365	grsB_1	Gramicidin S synthase 3	Secondary metabolite production	5.76	5.95
K659_RS0115225	BLU14_RS07365	ppsE_1	Plipastatin synthase subunit E	Secondary metabolite production	5.78	5.79
K659_RS0115230	BLU14_RS07370	macA	Macrolide export protein MacA	transporter activity	5.85	5.69
K659_RS0115235	BLU14_RS07375	macB2	Macrolide export ATP-binding/permease protein MacB 2	transporter activity	4.71	4.83
K659_RS0115245	BLU14_RS07385	oprM_1	Outer membrane protein OprM	transporter activity	4.39	4.29
K659_RS0115250	BLU14_RS07390	nccB	Nickel-cobalt-cadmium resistance protein NccB	stress response	3.52	3.83
K659_RS0115255	BLU14_RS07395	nolG_1	Nodulation protein NolG	transporter activity	2.50	2.59

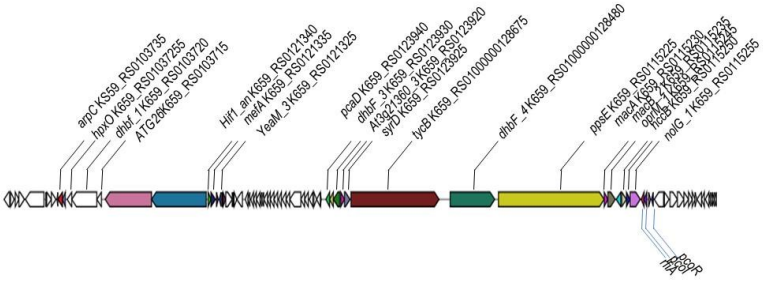

Figure 15. *P. corrugata* CFBP 5454 genes differentially expressed both in the mutant *pcoR* and *rfiA* that were localised in the biosynthetic cluster of CLPs as mined in *P. corrugata* type strain (black lines); QS and RfiA genes (blue lines).

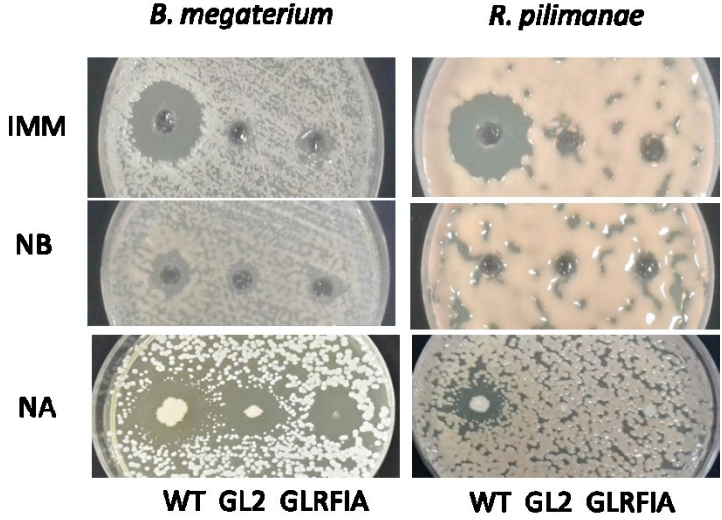

Figure 16. Antimicrobial assay to evaluate the bioactivity of *P. corrugata* WT, GL2 and GLRFIA cell-free culture filtrates grown in IMM and NB, and of cells against *B. megaterium* and *R. pilimanae.*

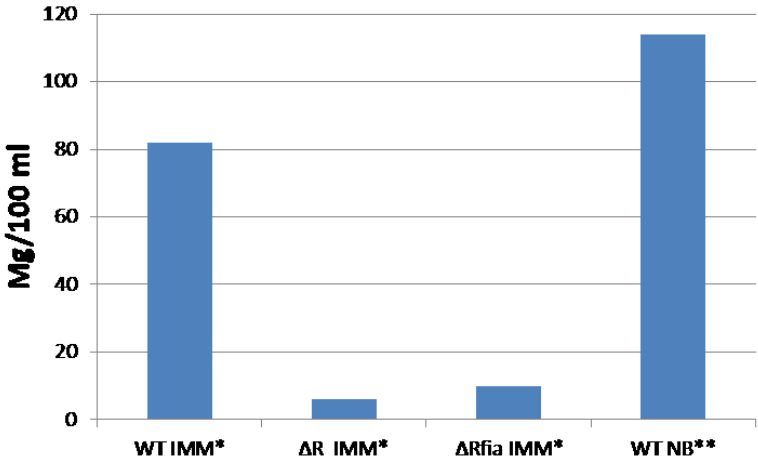

Figure 17. Yield of total exopolysaccarides harvested from the supernatant of *P. corrugata* CFBP5454 and GL2 and GLRFIA derivative mutants grown in IMM and NB to the early stationary phase, by KCL (25%) extraction, isopropanol precipitation and freeze-dried lyophylization.

4.3.3. Identification of genes regulated independently by PcoR and RfiA

Since the two mutant strains showed for some aspects different phenotypes, we have investigated also gene independently regulated by the PcoR and RfiA. The most relevant different concerns the presence of the *cyp4d2* gene, coding for cytochrome P450, involved in redox and oxidative stress, differentially expressed only in the GLRFIA mutant; it is downregulated in the Wt showing a LogFC -2.4 (Appendixes V-VI). We have to underline the strong upregulation of the *traI* gene, corresponding to *pcoI* gene, responsible of AHL synthase in the wild-type as compared to GL2 mutant. It confirm the correct functioning of the QS system since in the absence of the PcoR protein the transcription of the *pcoI* gene, strictly dependent on the formation of the PcoR-AHL complex in conditions of high cell density, such as that analyzed, does not occur. Among the genes differentially expressed in the GL2 mutant there are 6 downregulated transcriptional regulators although at low LogFC (-0.77 to 1.10). Three of them belongs to HTH transcriptional regulator family. In the GLRFIA there is only one HTH regulator downregulator (LogFC 1) and two upregulated.

4.3.4. Validation of the RNAseq expression patterns by quantitative real-time PCR (qPCR)

Following the initial analysis of the RNA-seq results, 13 of the PcoR- and RfiA-regulated genes involved in biosynthesis (six genes) and transport (seven genes) of non-ribosomal peptide biosynthesis were selected among the most highly regulated genes to validate RNA-seq results and qPCR was carried out with gene-specific primers (listed in

Table 6). The RNA-seq results were validated by the analysis of relative expression level of 13 selected genes co-regulated by PcoR- and RfiA by qPCR. A close correlation (Pearson's R^2 = 0. 7428) was observed between log_2FC measured by RNA-seq and qRT-PCR (Fig.18).
The six genes tested involved in NR peptide biosynthesis were *crpC, grsB_1, grsB_2, dhbF_3, dhbF_4* and *syrD2*, whereas those in NR peptide transport were *bepE_1, mefA, arpC, crpD, pcoA, nccB (pcoB)* and *opmR_3*. The RNAseq data trends for GL2 (*pcoR*-) and GLRFIA (*rfiA*-) were successfully validated via qPCR (Fig. 18). Although there was variability in the absolute values of the fold changes between the RNAseq and qPCR results, the trends of expression pattern were in good accordance. The data confirmed the positive regulation of PcoR and RfiA of all the selected genes, with the exception of *bepE_1* and *opmR_3*, which were downregulated in wild-type in comparison to both mutants , accordingly to RNAseq data.
The expression of *algD*, *algG* and *algI* genes analysed by qPCR confirm the upregulation in wild-type strain when compared to GL2 and GLRFIA mutants and consequentially the transcriptional control of PcoR and RfiA of alginate biosynthetic genes. Gene *cyp4d_2* was also included in the analysis as its opposite direction of regulation by PcoR and RfiA, confirming RNAseq data (data not shown).

4.3.5. CLP and Alginate gene expression analysis in different media and *in planta* by qPCR

Expression of NR peptide biosynthesis and transport and alginate genes was analysed in *P. corrugata* Wt strain grown in rich medium conditions (NB) and in plants in comparison to IMM minimal medium in order to define how the substrate can influence on CLPs and alginate production

activation. Results demonstrate that genes involved in NR peptide biosynthesis and transport are activated in minimal medium 2-6 fold more than in rich medium and with a less value also the genes involved in their transport (Fig. 19). This result demonstrates that these minimal conditions represent good conditions for CLP production.

In *planta* all the analysed genes, involved both in CLPs and alginate production resulted to be activated in comparison to growth on minimal conditions. Among the genes responsible of CLP bioysynthesis, the most activated are *dhbf_3* (12 fold) and *syrD2* (11,8 fold), whereas among the transporters *mefA* is 14,4 fold overexpressed. This confirms that CLPs production have a pivotal role in pathogenesis. The *algG* gene is also up-regulated in *planta* (12.8 fold) demonstrating that also alginate production can be associated to pathogenicity in *P. corrugata* (Fig. 19). Future mutagenesis analysis can confirms this data.

It is reported that alginate-producing strains naturally occurs among plant-associated *P. fluorescens* but certain phytopathogenic fluorescent *Pseudomonas* species are occasionally found to produce alginate both in plants and in vitro (Fett *et al.,* 1989, Fett *et al.,* 1986). It appears that many *Pseudomonas* species are capable of producing alginate but the genes involved in alginate biosynthesis are not normally expressed.

When we investigate on the production of EPSs in rich medium conditions (NB substrate) a higher yield has been obtained equal to 114 mg/100 ml in the Wt strain bacterial culture in comparison to Wt bacteria grown in IMM (82 mg/100 ml) showing that rich medium is a better condition for EPSs production.

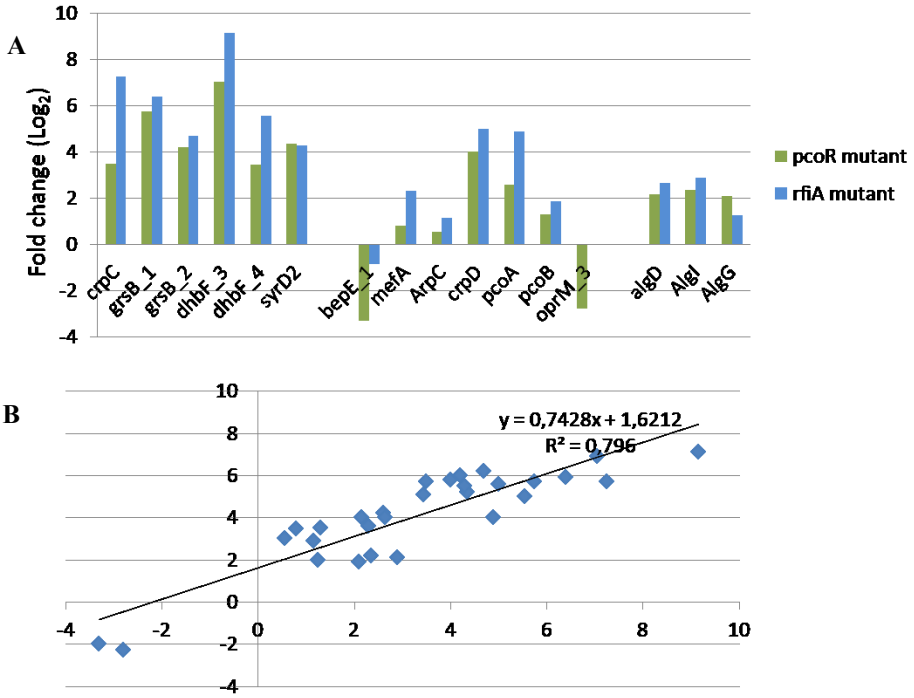

Figure 18. Validation by quantitative RT-PCR (qPCR) of transcriptional patterns of randomly selected genes involved in CLPs and alginate production. A) Transcriptional expression by qPCR in *P. corrugata* wild type strain in comparison to GL2 and GLRFIA mutants grown on IMM for 40 hrs at early stationary phase. The expression levels of all genes were standardized to the level of the constitutively expressed housekeeping 16s rDNA and normalized to expression in WT. The results represent the means and standard deviation (bars; some are not visible on this scale) of three independent experiments. B) Correlation of estimates fold change of differentially expressed transcripts between RNA-seq and qPCR analysis.

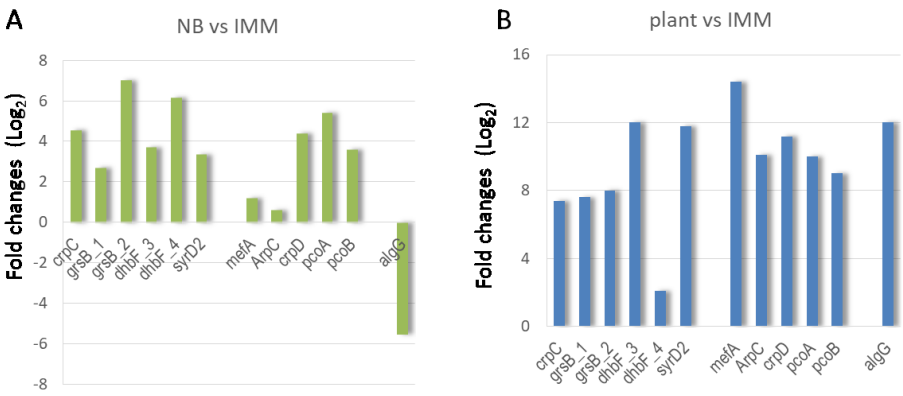

Figure 19. qRT-PCR showing the relative expression of a subset of genes from those validated involved in CLPs and alginate production in *P. corrugata* CFBP5454 WT grown in a rich medium i. e. Nutrient Broth (NB) (A) and in inoculated tomato plantlets (B) compared to the growth in IMM.

4.4. Antimicrobial activity of plant natural compounds against phytopathogenic bacteria and interference with quorum sensing

Bacterial diseases are difficult to control and Eu is planning to reduce commonly applied protective copper compounds. In this frame work, Eu is investing on bio-based pest management and plant health products for the agriculture. Different natural products could inhibit the growth of bacterial pathogens both *in vitro* than *in planta* (Montesinos, 2007; Pandey *et al.,* 2016). Plant essential oils produced by aromatic and medicinal plants were successfully tested against *X. vesicatoria*, *P. syringae* pv. *tomato*, *C. michiganansis* subsp. *michiganensis* and *R. solanacearum* (Pandey *et al.,* 2016).

Recently it has been demonstrated that plant phenols (e.g. coumaric acid, vannilin, cathecol) interfere with the QS mediated mechanism of virulence of phytopathogenic bacteria in the genus *Pectobaterium* (Joshi *et al.,* 2015). Among natural products, we focused on citrus industry by-products. They are essentially citrus peel and pulp; they are commonly used for livestock feed, for improve chemical and physic characteristic of soil and substrates and for producing biogas and bioethanol. Despite this diversity of usage, often the management of citrus peel and pulp represent a problem for citrus industries for disposal costs, and environmental problems.

In this study, we evaluated the antimicrobial activity of citrus peel components and phenols with relevant antioxidant activity, namely cathecol, citronellol, esperidin, limonene, quercitin and rutin, against nine phytopathogenic bacteria in the genera *Clavibacter*, *Erwinia, Pectobacterium*, *Pseudomonas* and *Xanthomonas*. In

addition we tested their possible QS – inhibition activity using a QS biosensor and *Pseudomonas corrugata* as model study.

4.4.1. Antimicrobial activity

In order to evaluate the bactericidal activity of solvents, bacterial strains representing the set of selected bacteria for testing were compared using two bacterial strains the Gram-negative *Pseudomonas syringae* pv. *tomato* strain PVCT28.31 and Gram-positive *Clavibacter michiganensis* subsp. *michiganensis* PVCT156.1.1. DMSO, unlike ethanol, methanol and acetonitrile, which inhibited bacterial growth with halos of about 0.1 to 0.3 cm, does not cause the formation of a bacterial growth inhibitory halo (data not shown). For this reason, the substances used in the studies conducted were diluted in DMSO. For the same reason DMSO was included as negative control in each replicate. Agar diffusion tests showed, after 24-48 hours of incubation, the activity of bacterial growth inhibition expressed by tested substances by the presence or not of inhibition halos around the paper disk adsorbed with the substance. In the same plate, the antibiotic activity of Gentamicin (positive control) and DMSO (negative control) was recorded for each bacterium.

Antimicrobial activity of substances against nine phytopatogenic bacterial are shown in Table 5. Limonene, cathecol and citronellol showed antimicrobial activity against all bacteria. No activity was observed by esperidin, quercitin and rutin. In general, antibacterial activity has been shown to be more effective against Xcc, Xv and Cmm Gram negative bacteria and Pch, Pst and Pcc Gram-positive bacteria. The highest inhibitory activity was induced by cathecol against *Xanthomonas* species and *P. syringae* pv.

tomato and by citronellol against *C. michiganensis* subsp. *michiganensis* and *E. amylovora* (Fig. 20). Weak or no activity was recorded for the other compounds (data not shown).
In particular the inhibition halo of cathecol ranged from a minimum of 1.3 cm against Ea to a maximum of 3.4 cm against Xcc and Xv. The inhibition halo of citronellol ranges from a minimum of 0.7 cm against Pcc to a maximum of 2.4 cm in Cmm.
The interference of the substances with the kinetic bacterial growth of nine phytopathogenic bacteria was further evaluated by Bioscreen C (Labsystems, Helsinki, Finland) and absorbance measurements performed for 48 h. MIC ranged from 0.5 to 0.0625 mg mL^{-1} and 1 to 0.125 mg mL^{-1}, for cathecol and citronellol, respectively (Fig. 21).

4.4.2. Quorum sensing inhibition

In order to determine if any substance can target the classical AHL based QS signal molecules, a range of QS inhibition experiments were performed.
In a first approach, the potential Quorum sensing-inhibition (QS-I) activity of the five compounds was assessed by using the reporter strain *Chromobacterium violaceum* ATCC 31532. CV is a Gram negative bacteria, his colonies are purple due to the production of violacein which is regulated by AHL-QS. The presence of white colonies near the tested compound was recorded as Quorum Quencing (QQ). Of all the tested substances, only citronellol, despite having shown less antagonistic activity than catechol, revealed QQ activity against the tested biosensor at different concentration, causing a lack of pigmentation of typically purple *C. violaceum* colonies at the margin of the inhibition halos (Fig. 22). To confirm that the reduction of pigment

production was related to the inhibition of the QS system, and not any growth defect, re-isolation of the biosensor strain from the inhibition spots resulted in characteristic pigment production after 24 h of growth in the absence of citronellol (data not shown).

In order to determine the quorum quenching activity of citronellol against AHLs we also used the QS reporter strain *C. violaceum* CV026. CV026 is a white colony mutant that could be used as biosensor able to respond to a series of synthetic AHLs which induce violacein production; this change could be inhibited in presence of QQ compounds. Citronellol at 0.5, 0.25 and 0.125 mg was able to inhibit QS system-associated pigment production in CV026 when AHLs were added to the well (Fig. 23). Citronellol at all concentrations displayed a similar inhibition effect against the medium chain AHLs, C6-HSL and C8-HSL. These results suggested that citronellol could have an important role against AHL-QS signal molecules.

4.4.3. Interference of citronellol with the QS system of *P. corrugata*

Interference with Quorum sensing

In order to determine if citronellol interferes with the transcription of AHL-synthase gene, *pcoI*, in the formation of PcoR-AHL complex, we tested *pcoI* promoter activity through a ß-galactosidase assay. The promoter region of *pcoI* has been cloned in the broadhost-range low-copy-number ß-galactosidase promoter probe vector pMP220, yielding pMPPcoI. Promoter activity was determined in response to the growth phase of the WT strain CFBP 5454 and GL1 (*pcoI*) mutant derivative, unable to synthetize AHL. With the aim to detect the best time to detect ß-galactosidase activities, we have preliminarly measured

population densities (OD_{600}) at 18, 24 and 42 hours. Detection at 24 hrs has been selected because all the cultures reached a population density of 10^8 CFU mL^{-1} able to activate AHL-QS system.

As expected, *pcoI* promoter activity was undetectable in the *pcoI P. corrugata* mutant GL1 (*pMPPcoI*) (157 Miller Units), showing a 90% reduction in comparison to Wt (1132 Miller Units), indicating that it was under positive autoregulation, as is often the case with the luxI/R systems.

Exogenous addition of C6-HSL (5 µM), the most active signal molecule produced by *P. corrugata* CFBP 5454 (Licciardello *et al.,* 2007), to the *pcoI* GL1 mutant harboring pMPPcoI restored promoter activity (900 Miller Units) (Fig. 24).

Exogenous addition of citronellol reduced the ß-galactosidase activity depending on concentration. It was higher when citronellol was added at 12.5 mg mL^{-1} concentration (874,9 Miller Units) than at 50 mg mL^{-1} (155 Miller Units) (Fig. 24). No differences were obtained after addition of DMSO (negative control) in comparison to GL1 (*pMPPcoI*) in presence of C6-HLS (812 Miller Units) (Fig. 24).

Effect of citronellol on CLP production in P. corrugata

P. corrugata strain CFBP 5454 possess an AHL-QS systems, designated PcoI/PcoR, which relies on two proteins, an AHL synthase, and a transcriptional regulator. PcoI/PcoR regulates the production of the phytotoxic and antimicrobial cyclic lipopeptides (CLPs) corpeptins and cormycin (Licciardello *et al.,* 2012). Main AHLs produced by *P. corrugata* are C6-HSL and C8-HSL (Licciardello *et al.,* 2007), thus in the same range of those produced by *C. violaceum* and those to which in the interference mediated by citronellol was observed in our trials.

To determine if the QS-Inhibition was linked to an anti-virulence activity, the effect of citronellol was tested on cultures of *P. corrugata.* Phenotypic effect on CLP production was evaluated by testing the antimicrobial activity against the two CLP bioindicators, the yeast *Rhodotorula pilimanae* ATCC26423 and the Gram-positive bacterium *Bacillus megaterium* ITM100. Citronellol reduced both CLP production, measured as antimicrobial activity of culture filtrates on bioindicators; the cultural filtrates obtained from *P. corrugata* cultures treated with citronellol showed a decrease or a loss of the inhibition zone compared with the control depending on the concentration of citronellol (Fig. 25).

4.4.4. CLP Gene expression

To further characterize the response of *P. corrugata* CFBP5454 to citronellol, we also analysed the expression of genes contributing to production (biosynthesis) of CLPs *grsb_2* and *ppsE* and their regulator PcoI/PcoR. These genes are located downstream of the QS cascade and are considered to be QS dependent (Licciardello *et al.,* 2012). The expression of *grsb_2* and *ppsE* genes, in the citronellol treated cultures shows significantly lower expression levels relative to controls after 96 h (Fig. 25). The results, shown in Fig. 25, demonstrate substantial concentration-dependent downregulation of both genes. Expression of *ppsE* appeared to be inhibited substantially more strongly than that of *grsb_2*. The data are based on the results of three independent experiments. It is not possible to establish a significant difference from the standard deviation of the results.

Tested natural compounds, contained in industrial citrus wastes, showed a direct effect on bacterial growth of plant

pathogenic bacteria and those effective such as cathecol and citronellol could be further exploited for disease control methods. Citronellol showed a marked QS- inhibition activity, interfereing with the QS AHL signal of the biosensor strain *C. violaceum*. The treatments with non-lethal concentration of citronellol on the model pathogen *P. corrugata* enabled studying its effect on phytotoxic CLP production which appear reduced if not impaired in treated culture.

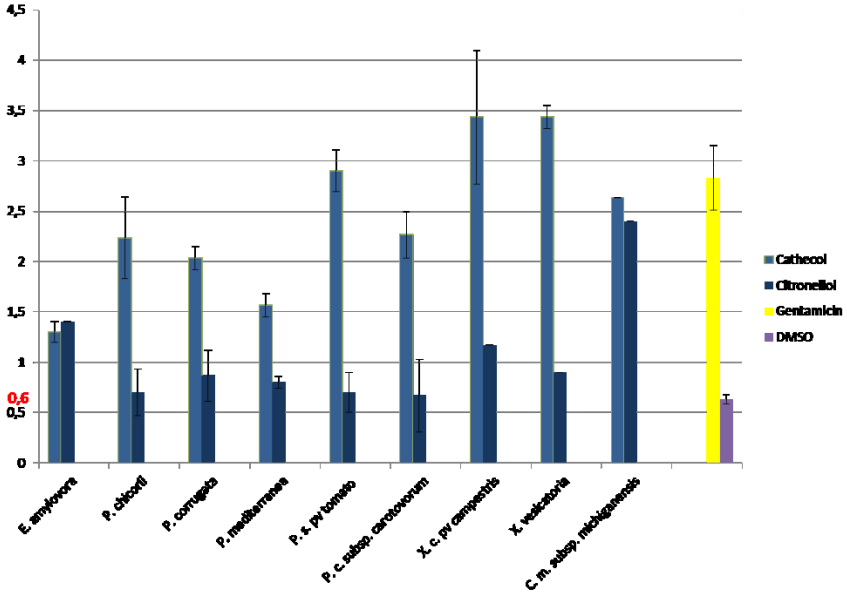

Figure 20. Disc diffusion assay**.** Average values inhibition diameters (cm) as clear zones around the test discs treated with catechol and citronellol with a concentration of 100 mg/ml against Gram-negative and Gram-positive phytopathogenic bacteria (all tests were performed in triplicate); gentamicin 10 µg and DMSO were used as positive and negative control, respectively (mean of control discs for each bacterial species). The dotted line represents the diameter of the paper disk (0,6 cm).

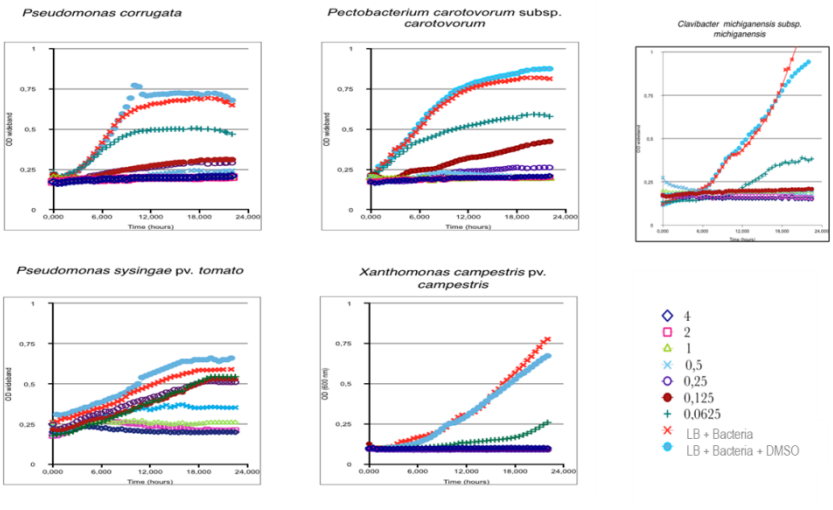

Figure 21. Broth diluition method. Growth curves of *P. corrugata, P. syringae* pv. *tomato, P. carotovorum* subsp. *carotovorum, X. campestris* pv. *campestris, C. michiganensis* subsp. *michiganensis bacterial* strain obtained by continuos growth in Luria broth (LB) medium with different concentration of citronellol as indicated in figure (mg/ml) using Bioscreen C. Each graph has indicative control curves for bacterial growth without citronellol or added with DMSO.

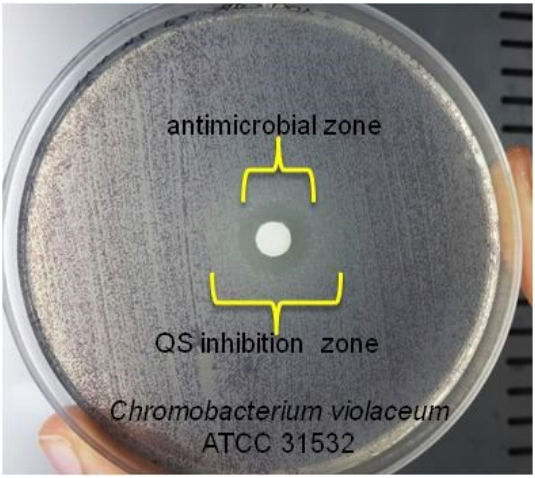

Figure 22. Quorum quenching activity of citronellol. Different amount of citronellol were spotted onto sterile paper disk in a plate inoculated with *C. violaceum* ATCC 31532. Citronellol both inhibit growth and violacein production near the disc zone.

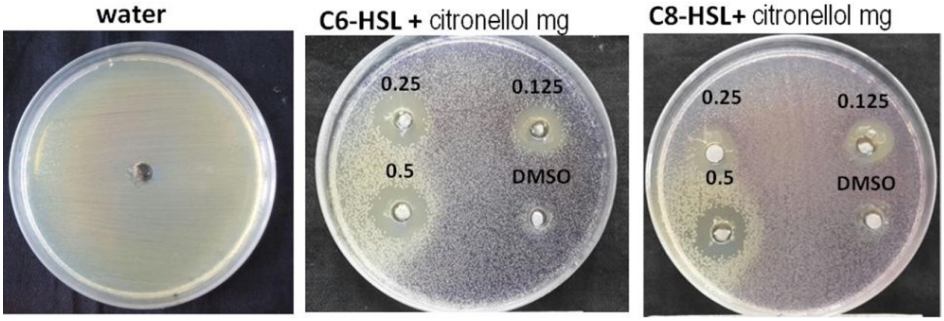

Figure 23. Citronellol activity against AHLs. The quorum sensing biosensor strain *C. violaceum* CV026 was used to detect citronellol activity against medium AHLs. LB agar plates were inoculated with *C. violaceum* CV026 (OD_{600} 0.5). Wells made in the agar plates were filled with 20 µL of 200 µM concentration of C6-HSL and C8-HSL and 10 µL of citronellol at different concentration (0.5; 0.25 and 0.125 mg/ml) . To determine the effect of citronellol against long AHLs DMSO was used as control.

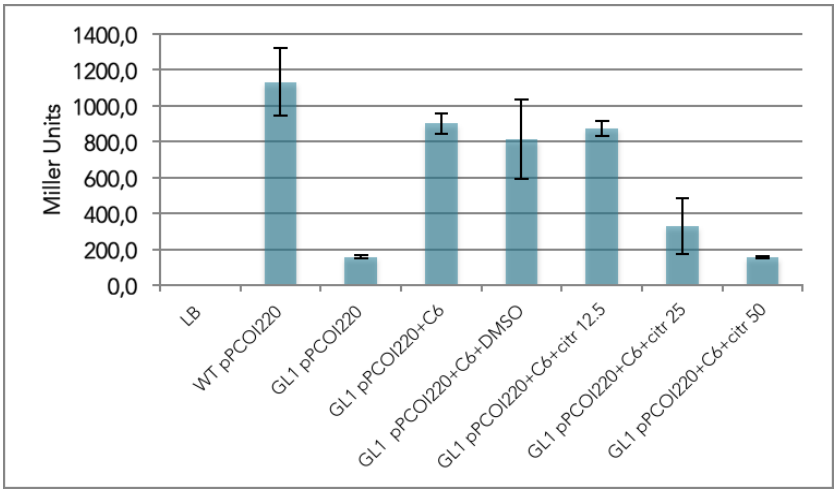

Figure 24. Evaluation of β-galactosidase activity of WT strain and GL1 mutant strain in presence of exogenous AHL, DMSO and different concentration of citronellol (LB represent the negative control).

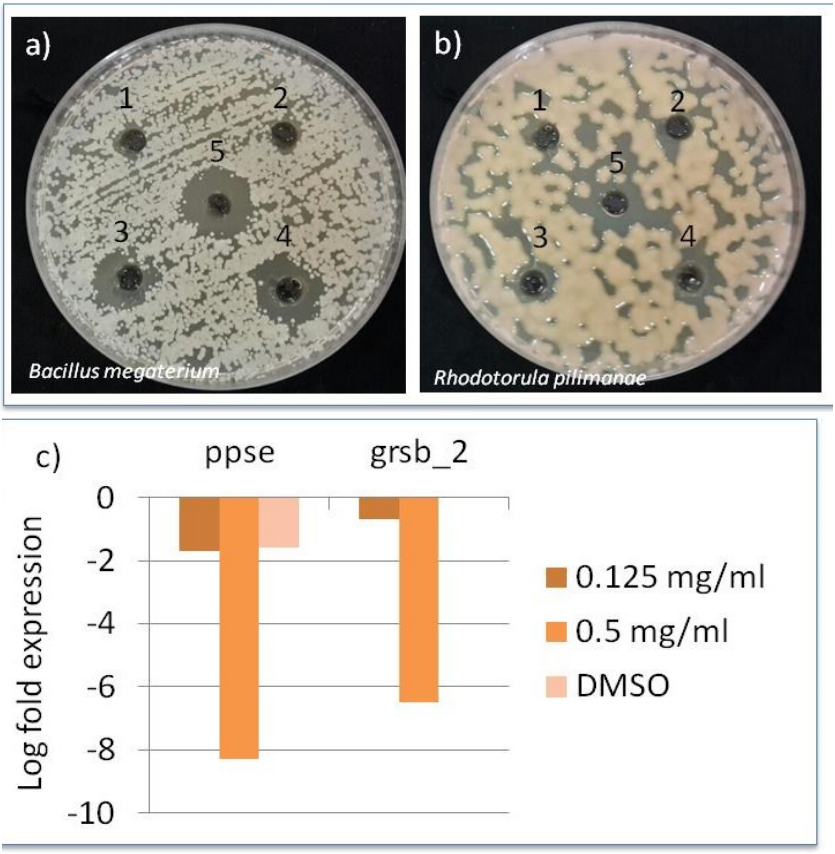

Figure 25. CLP production assay: antagonistic activity of culture filtrates of the bacterium *P. corrugata* strain CFBP5454 grown in IMM medium with different citronellol concentration against the CLP indicator strains *Bacillus megaterium* **(a)** and *Rhodotorula pilimanae* **(b)**. 1- 0,125/ 2- 0,0625/ 3- 0,312/ (mg/ml)4 – DMSO 5- non treated bacteria.
(c) Relative expression of genes *crpC* and *grsb_2* involved in corpeptin and cormycin biosynthesis, respctively by qPCR. RNA samples were extracted from three biological replicates of each thesis by using the RNeasy Mini Kit (Qiagen). RNA was used for cDNA synthesis with Superscript III (Invitrogen) according to the manufacturer's protocol. Relative expression data were obtained by the ΔΔCT method. All the Ct values were normalized to the 16S ribosomal RNA gene.

5.Discussion

We investigated the model strain *P. corrugata* CFBP 5454, to shed light on the determinants involved in the control of antibiosis. *P. corrugata* has a peculiar AHL-QS system PcoI/PcoR that in turn regulates the *rfiA* gene that encode a LuxR transcriptional regulator that lack the AHL binding domain (Licciardello *et al.,* 2007; 2009). Thus, PcoR activates *pcoI* expression in the presence of AHL via a typical positive-feedback regulatory loop and as *pcoI* and *rfiA* constitute an operon, the expression of *rfiA* is directly regulated by the PcoR–AHL complex (Licciardello *et al.,* 2009).

Distinctive phenotypes were observed for *P. corrugata* CFBP5454 *pcoR* and *rfiA* knock out mutants, but not for the mutant in *pcoI* encoding the AHL-synthase. They did not produce corpeptins and cormycin and they have reduced antagonistic activity against phytopathogenic fungi and bacteria (Licciardello *et al.,* 2009, 2012, Strano *et al.,* 2017) and have a role in the expression of genes belonging to the corpeptin biosynthesis cluster (*crpC* and *crpD*) (Strano *et al.,* 2015).

Our results showed that *P. corrugata* CFBP 5454 produced diffusible compounds, which have the ability to inhibit the in vitro growth of plant pathogenic fungi and bacteria. *P. corrugata* antimicrobial activity has often been attributed to the production of the CLP corpeptins (Emanuele *et al.,* 1998) and cormycin A (Scaloni *et al.,* 2004), and to the siderophore corrugatin (Risse *et al.,* 1998). The antimicrobial activity observed on PDA against the Gram-positive bacterium *C. michiganensis* subsp. *michiganensis*, appeared greater than against the Gram-negative *P. syringae* pv. *tomato.* Presumably, the reduced or null activity of the diffusible compounds produced by CFBP 5454 strain

against Gram-negative bacteria could be ascribed to the protective effect 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, has 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). In culture, *P. corrugata* strain CFBP 5454 produce both corpeptins and the nonapeptide cormycin (Licciardello *et al.,* 2012). 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).

In a previous study we observed that the cell-free culture filtrates of *P. corrugata* GL2 (pcoR mutant) and GLRFIA (rfiA mutant) strains, did not contain corpeptins and cormycin (Licciardello *et al.,* 2012). In tests against CLP bioindicator strains, we found that the 10× concentrate cell-free culture filtrates of all the above-mentioned mutants, no longer produced antimicrobial activity. Nevertheless, tests using bacterial cells highlighted that supplementary antimicrobial metabolites are produced by both bacterial strains.

This is consistent with the fact that the GL2 (pcoR mutant) strain was still able to inhibit the growth of both *R. pilimanae* and *B. megaterium*; whereas the activity against the yeast was impaired in the *P. corrugata* GLRFIA (rfiAmutant) strain. In addition, the failure of these latter mutant strain to inhibit fungi was also confirmed against *A. niger*, *B. cinerea*, *P. digitatum* and *P. tracheiphila*.

The results thus suggest that at least two other metabolites are produced by GL2 (pcoR mutant), one targeting fungi

regulated by RfiA and one antibacterial substance. Indeed, when inoculated in tomato plantlets, *P. corrugata* GLRFIA (rfiA mutant) strain, a drastic reduction in virulence was found without necrosis in the inoculated stems (Licciardello *et al.,* 2009).

Some authors have reported the production of several secondary metabolites for *P. corrugata* strains. The genes putatively involved in their production have in many cases been detected and the sequences deposited in the GenBank. 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) have been described, but none of these aspects have been investigated further.

Therefore, *P. corrugata* and biocontrol activity is mediated by an array of diffusible compounds ascribable to the CLPs cormycin and corpeptins and other diffusible antimicrobial compounds. Based on the genome analysis results of Trantas *et al.* (2015 a), at least two other NR-peptides, a polyketide and two bacteriocines are putatively produced by the two bacterial species. Numerous studies have demonstrated that metabolites, including antibiotics, enzymes, and volatiles produced by soil- and plant-associated bacteria, are key factors in the suppression of plant pathogens (Haas and Défago, 2005; Raaijmakers and Mazzola, 2012; Loper *et al.,* 2012,), and several microorganisms have been commercially developed as biopesticides.

In addition, antimicrobial peptides, which are currently being tested for pharmaceutical purposes, could play a strong role in agriculture as plant protection products or as biopesticide active substances or for modeling more active

synthetic peptides (Montesinos, 2007; Raaijmakers and Mazzola, 2012). Although antibiotics most likely do not act in isolation but work together with other metabolites produced by the same cell, we are currently investi-gating whether more compounds could be produced in different growing conditions.

We therefore performed a transcriptome study to unravel the PcoR and RfiA regulons resulting in a global picture of their role in *P. corrugata* biology. Our results showed that PcoR and RfiA co-regulates the 3% of the annotated genes in the CFBP5454 draft genome. The results showed that very important genes coding for secondary metabolites are part of the PcoR-RfiA regulon, namely genes involved in the biosynthesis of antimicrobial LPs and those for the production of alginic acid. *P. corrugata* produces the CLPs corpeptins, two isoforms consisting of 22-amino-acid residues, corpeptin A and corpeptin B. Corpeptins induce chlorosis when infiltrated into tobacco leaves and show antimicrobial activity against the Gram-positive bacterium *Bacillus megaterium* (Emanuele *et al.,* 1998).

Some strains also produce the nonapeptide cormycin A, which has antimicrobial activity against *B. megaterium* and also against the yeast *Rhodotorula pilimanae* and phytotoxic activity (Scaloni *et al.,* 2004). *P. corrugata* strain CFBP5454 produces both CLPs (Licciardello *et al.,* 2012). Like many other biologically active secondary metabolites, CLPs are synthesized by multifunctional non-ribosomal peptide synthetases (NRPSs) (Raaijmakers *et al.,* 2006). It is estimated that approximately 3 kb of DNA are required to code for each amino acid activation module (Gross and Loper, 2009); thus due to the incomplete nature of the *P. corrugata* strain CFBP5454 draft genome the large CLP

NRPs are divided over different contigs (Licciardello *et al.,* 2014; Trantas *et al.,* 2015 a).

Combining BLAST analysis of gene down-regulated in *P. corrugata* CFBP5454 *pcor* and *rfiA* mutants and genome mining of the whole genome sequence of strain LMG 2172^{T} in the GenBank repository we ascertained that 19 differentially regulated genes were located in a large cluster accounting for approximately the 3.4 % of the genome. This DNA region includes the gene clusters for cormycin and corpeptin and a putative brabantamide-like gene cluster down-stream of the putative cormycin gene cluster as well as the genes of the QS-RfiA system downstream the corpeptin cluster.

This organization is consistent with the gene organization present in the biocontrol strain *Pseudomonas* SH-C52 for the thanapeptins, thanamycin and brabantamide lipopeptides (Van der Voort *et al.,* 2015). Also *P. syringae* pv *syringae* and *P. fluorescens* In5 produce both CLPs characterized by long peptide chains and smaller nonapeptides and their biosynthesis clusters are adjacently located in the genome as the case of syringopeptins and syringomycin (Scholz-Schroeder *et al.,* 2001) and nunamycin and nunapeptins, respectively (Henessy *et al.,* 2017). In particular, PcoR-RfiA regulates the genes for the three NRPSs necessary for biosynthesis of corpeptins and the downstream located ABC transporter. Part of these were described by Strano *et al.* (2015) that designated *crpCDE* genes transcriptionally joined coding for an NRPS and an ABC efflux system.

The introduction of a mutation in *crpC* yielded a *P. corrugata* strain, PCONRPS, which failed to produce corpeptins, thus demonstrating that *crpC* is part of the corpeptin biosynthesis locus. Gene disruption of *crpD* also affected the presence of corpeptins in the culture filtrates of

P. corrugata CFBP5454, supporting the assumption that CrpDE is the unique transport system involved in corpeptin export.

Although the *pcoR* and *rfiA* mutants grown in the same conditions as in this study also failed to produce cormycin (Licciardello *et al.,* 2012), none of the cormycin NRPS genes was evidenced among the differentially expressed genes. Nevertheless, both a putative ABC transporter system highly homologous to trasporters for syringomycin or thanamycin in *P. syringae* B301D (Kang and Gross, 2005), B728A (Vaughn and Gross, 2016) and in *Pseudomonas* SHC52 (Van der Voort *et al.,* 2015) and a gene annotated as *syrD_2,* that in *P. syringae* B301D has been shown to play a role in the secretion of syringomycin and syringopeptin (Quigley *et al.,* 1993), was regulated by PcoR and RfiA.

Van der Vort *et al.* (2015) already described in the genome of *P. corrugata* the presence of a gene cluster putatively involved in the synthesis of brabantamide described in *Pseudomonas* sp. SHC52 (Van Der Voort *et al.,* 2015). Brabantamide is 2-aminoacid-lipopeptide containing a glycosylated 3-hydroxy fatty acid tail, already described in *Pseudomonas* strain SH-C52, which show antimicrobial activity against Gram-positive bacteria and Oomycetes. Four genes of the brabantamide biosynthesis cluster are also in the PcoR-RfiA regulon.

Phenotypic assay support our data being culture filtrates depleted of the antimicrobial activity although tests performed using living cultures still have antimicrobial activity. This evidence suggests that more secondary metabolites that are not regulated by the PcorR-RfiA regulon are involved. Accordingly, Trantas *et al.* (2015 a) several secondary metabolites gene cluster were found in the genomes of *P. corrugata* strain CFBP5454 and other three

strains among them putative clusters for an additional NR peptide, a polyketide and a bacteriocin. In the genome of *P. corrugata* strain LMG 2172^T seven secondary metabolite clusters were mined (Supplemental file 5) accounting for approximately the 7.2 % of the genome (data not shown).
Pseudomonas CLP biosynthesis clusters are flanked by genes coding for LuxR transcriptional regulators (reviewed in Raaijmakers *et al.,* 2010) but up to recently the presence of a LuxR regulator directly linked to an AHL-QS system by mean of gene cotranscription with acyl-homoserine lactone synthase gene was described only for *P. corrugata* and *P. mediterranea* (Licciardello *et al.,* 2012). Trantas *et al.* (2015 a) found the QS-RfiA system also in the genomes of *P. corrugata* strains CFBP5403, TEIC 1148, NCPPB 2445^T as well as in five strains of the closely related species *P. mediterranea*. This system is conserved also in other *P. corrugata* and *P. mediterranea* genomes recently included in the GenBank repository and in strain *Pseudomonas* sp. SCH52. *P. corrugata* CFBP5454 PcoI, PcoR and RfiA showed protein homologies of the 100% with the corresponding proteins in *P. corrugata* strain LMG 2172^T, of 85% 95% and 94% with strain *Pseudomonas* sp. SHC52, and of 84%, 95% and 92% with *P. mediterranea* strain DSM16733^T, respectively (data not shown).
PcoR and RfiA have also a role in regulation of alginic acid biosynthesis gene expression. In particular, all the genes belonging to the structural/biosynthetic cluster of the exopolysaccharide alginate were upregulated in the wild type as compared to the GL2 and the GLRFIA mutant. Screening studies of EPSs production showed that three *P. corrugata* strains (388, 717, and ATCC 29736^T) produced EPSs after growth on Pseudomonas Agar F (PAF) with 5% glycerol (Fett *et al.,* 1996). EPS produced by *P. corrugata*

388 are constituted of solely uronic acid (100% w/v) and after reduction and GLC analysis the presence of mannose alone has been detected (Fett *et al.,* 1996). Although, Fett *et al.* (1992) described that *P. corrugata* strain 388 contain genes with homology to alginate biosynthetic and regulatory genes, the gene cluster was not investigated in the *P. corrugata* genome studies (Licciardello *et al*., 2014; Trantas *et al.,* 2015 a). However, it was described in the closely related species *P. mediterranea* (Licciardello *et al.,* 2016) and in *Pseudomonas* sp. SH-C52 genomes (Van der Vort *et al.,* 2015). The analysis of *P. corrugata* CFBP 5454 genome revealed that, similarly to other *Pseudomonas* spp. that belong to rRNA homology group I (Fett *et al.,* 1992), the alginate biosynthesis and regulatory genes are widely distributed into three clusters namely, structural/biosynthetic, regulatory and genetic switching genes (Table 3; Fig. 6). A similar cluster was highlighted in other *P. corrugata* genomes as well as in other species of the *P. corrugata* clade within the *P. fluorescens* group (data not shown).

According to *P. aeruginosa* studies, the core genes involved in alginate biosynthesis are in a single 12-gene operon initially described by Chitnis and Ohman (1993) including *algD*, *alg8*, *alg44*, *algK*, *algE*, *algG*, *algX*, *algL*, *algI*, *algJ*, *algF*, *algA* and *algC*. The genes are under the tight control of a promoter upstream of *algD* (Schurr *et al.,* 1993; Shankar *et al.,* 1995), although there is some evidence to suggest that alternative internal promoters exist within the operon (Lloret *et al.,* 1996; Paletta and Ohman, 2012). The production of EPS by plant-pathogenic bacteria is considered to be an important strategy that contributes significantly to their growth, survival, and virulence (Lindow and Brandl, 2003; Von Bodman *et al.,* 2003). EPS

production in *Pantoea stewarti, Ralstonia solanacearum, Xanthomonas campestris* is subject to regulation by QS (Von Bodman *et al.,* 2003). Moreover, it has been demonstrated that in *P. syringae* B728a quorum sensing contributes to the production of alginate, a major component of EPS (Quinones *et al.,* 2005).

Results in the overall suggest *P. corrugata* QS-RfiA system regulates hierarchically by PcoR and RfiA transcriptional regulators important secondary metabolite production at high cell concentration. It is interesting that *P. corrugata* is known as phytopathogenic bacterial species but many studies look at it as a biological control agent and both antimicrobial peptide and alginate are strongly linked to bacterial fitness of plant associated bacteria in interaction with other microorganisms as well as plants.

Many bacterial species/strains within the *P. corrugata* subgroup in the *P. fluorescens* group, are described as biocontrol agent such as *P. brassicacearum* subsp. *brassicacearum* NFM421T, *P. fluorescens* F113 and *Pseudomonas* sp. SC-H52 (Trantas *et al.,* 2015 a). In addition, *P. corrugata* (and the closely related species *P. mediterranea*) lack a Type III Secretion System and related effectors characteristic feature of plant pathogenic *Pseudomonas*. In this respect, it would be interesting in future studies to focus on the identification of *in vivo* stimuli that could allow to this bacterium to switch from a resident phase in the rhizosphere or as endophyte to the pathogenic phase.

6. Appendixes

Appendix I - Antismash Cluster organization of secondary metabolites for NZ_LT629798.1

Gene cluster type	Gene cluster genes	Gene cluster gene accessions
CLUSTER 1 hserlactone-nrps	BLU14_RS07090;BLU14_RS07095;BLU14_RS07100;BLU14_RS07105;BLU14_RS07110;BLU14_RS07115;BLU14_RS07120;BLU14_RS07125;BLU14_RS07130;BLU14_RS07135;BLU14_RS07140;BLU14_RS07145;BLU14_RS07150;BLU14_RS07155;BLU14_RS07160;BLU14_RS07165;BLU14_RS07170;BLU14_RS07175;BLU14_RS07180;BLU14_RS07185;BLU14_RS07190;BLU14_RS07195;BLU14_RS07200;BLU14_RS07205;BLU14_RS07210;BLU14_RS07215;BLU14_RS07220;BLU14_RS07225;BLU14_RS07230;BLU14_RS07235;BLU14_RS07240;BLU14_RS07245;BLU14_RS07250;BLU14_RS07255;BLU14_RS07260;BLU14_RS07265;BLU14_RS07270;BLU14_RS07275;BLU14_RS07280;BLU14_RS07285;BLU14_RS07290;BLU14_RS07295;BLU14_RS07300;BLU14_RS07305;BLU14_RS07310;BLU14_RS07315;BLU14_RS07320;BLU14_RS07325;BLU14_RS07330;BLU14_RS07335;BLU14_RS07340;BLU14_RS07345;BLU14_RS07350;BLU14_RS07355;BLU14_RS07360;BLU14_RS07365;BLU14_RS07370;BLU14_RS07375;BLU14_RS07380;BLU14_RS07385;BLU14_RS07390;BLU14_RS07395;BLU14_RS07400;BLU14_RS07405;BLU14_RS07410;BLU14_RS07415;BLU14_RS07420;BLU14_RS07425;BLU14_RS07430;BLU14_RS07435;BLU14_RS07440;BLU14_RS07445;BLU14_RS07450;BLU14_RS07455;BLU14_RS07460;BLU14_RS07465;BLU14_RS07470;BLU14_RS07475;BLU14_RS07480;BLU14_RS07485;BLU14_RS07490	WP_024776975.1;WP_024776974.1;WP_024776973.1;WP_024776972.1;WP_053191113.1;WP_024776970.1;WP_050593780.1;WP_053191115.1;WP_024776967.1;WP_024776966.1;WP_024776965.1;WP_024776964.1;WP_053191118.1;WP_053191119.1;WP_053191120.1;WP_053191121.1;WP_024780237.1;WP_024780236.1;WP_024780235.1;WP_024780234.1;WP_024780233.1;WP_053191123.1;WP_053191125.1;WP_053191127.1;WP_041024143.1;WP_024780228.1;WP_053191133.1;WP_024780226.1;WP_024780225.1;WP_053191135.1;WP_053191137.1;WP_053191139.1;WP_024780221.1;WP_024780220.1;WP_024780219.1;WP_053191140.1;WP_053191142.1;WP_053191143.1;WP_024780215.1;WP_053191145.1;WP_053191147.1;WP_053191150.1;WP_024780211.1;WP_053191152.1;WP_053191154.1;WP_024780208.1;WP_024780207.1;WP_024780206.1;WP_024780709.1;WP_024780708.1;WP_053191156.1;WP_024780706.1;WP_053191158.1;BLU14_RS07355;WP_053193696.1;WP_081357976.1;WP_024779109.1;WP_053191162.1;WP_053191164.1;WP_024779112.1;WP_080996558.1;WP_053191166.1;WP_024779115.1;WP_024779116.1;WP_053191168.1;WP_024779118.1;WP_053191170.1;WP_053191173.1;WP_053191175.1;WP_053191176.1;WP_053191177.1;WP_053191179.1;WP_053191182.1;WP_053191183.1;WP_053191185.1;WP_053191187.1;WP_024779129.1;WP_024779130.1;WP_024779131.1;WP_053191189.1;WP_053191191.1

Appendix I - Antismash Cluster organization of secondary metabolites for NZ_LT629798.1

Gene cluster type	Gene cluster genes	Gene cluster gene accessions
CLUSTER 2 siderophore	BLU14_RS09365;BLU14_RS09370;BLU14_RS09375;BLU14_RS09380;BLU14_RS09385;BLU14_RS09390;BLU14_RS09395;BLU14_RS09400;BLU14_RS09405;BLU14_RS09410;BLU14_RS09415;BLU14_RS09420;BLU14_RS09425;BLU14_RS09430	WP_053191794.1;WP_053191792.1;WP_024777662.1;WP_024777663.1;WP_053191888.1;WP_053191790.1;WP_024777666.1;WP_053191788.1;WP_053191886.1;BLU14_RS09410;WP_053191787.1;WP_053191785.1;WP_053191783.1;WP_053191781.1
CLUSTER 3 t1pks	BLU14_RS10070;BLU14_RS10075;BLU14_RS10080;BLU14_RS10085;BLU14_RS10090;BLU14_RS10095;BLU14_RS10100;BLU14_RS10105;BLU14_RS10110;BLU14_RS10115;BLU14_RS10120;BLU14_RS10125;BLU14_RS10130;BLU14_RS10135;BLU14_RS10140;BLU14_RS10145;BLU14_RS10150;BLU14_RS10155;BLU14_RS10160;BLU14_RS10165;BLU14_RS10170;BLU14_RS10175;BLU14_RS10180;BLU14_RS10185;BLU14_RS10190;BLU14_RS10195;BLU14_RS10200;BLU14_RS10205;BLU14_RS10210;BLU14_RS10215;BLU14_RS10220;BLU14_RS10225;BLU14_RS10230;BLU14_RS10235;BLU14_RS10240;BLU14_RS10245;BLU14_RS10250;BLU14_RS10255	WP_024780000.1;WP_024779999.1;WP_024779998.1;WP_024779997.1;WP_053191697.1;WP_024779995.1;WP_053191695.1;WP_024779993.1;WP_053191694.1;WP_024779991.1;WP_053191692.1;WP_024779989.1;WP_024779988.1;WP_024779987.1;WP_053191690.1;WP_081357977.1;WP_081357978.1;WP_080996574.1;WP_024779983.1;WP_024779982.1;WP_024779981.1;WP_024779980.1;WP_024779979.1;WP_053191685.1;WP_024779977.1;WP_024779976.1;WP_024779975.1;WP_053191422.1;WP_024780835.1;WP_053192481.1;WP_053192482.1;WP_053192485.1;WP_024780831.1;WP_024780830.1;WP_024780829.1;WP_024780828.1;WP_024780827.1;WP_053192487.1
CLUSTER 4 nrps	BLU14_RS11035;BLU14_RS11040;BLU14_RS11045;BLU14_RS11050;BLU14_RS11055;BLU14_RS11060;BLU14_RS11065;BLU14_RS11070;BLU14_RS11075;BLU14_RS11080;BLU14_RS11085;BLU14_RS11090;BLU14_RS11095;BLU14_RS11100;BLU14_RS11105;BLU14_RS11110;BLU14_RS11115;BLU14_RS11120;BLU14_RS11125;BLU14_RS11130;BLU14_RS11135;BLU14_RS11140;BLU14_RS11145;BLU14_RS11150;BLU14_RS11155;BLU14_RS11160;BLU14_RS11165;BLU14_RS11170;BLU14_RS11175;BLU14_RS11180;BLU14_RS11185;BLU14_RS11190;BLU14_RS11195;BLU14_RS11200;BLU14_RS11205;BL	WP_024779817.1;WP_024779816.1;WP_024779815.1;WP_024779814.1;WP_053193361.1;WP_053193362.1;WP_053193367.1;WP_050593886.1;WP_053193368.1;WP_053193370.1;WP_053193373.1;WP_024780739.1;WP_024780740.1;WP_053193378.1;WP_053193381.1;WP_080996602.1;WP_053193383.1;WP_053193385.1;WP_053193387.1;WP_053193389.1;WP_053193391.1;WP_081357981.1;BLU14_RS11145;WP_053193707.1;WP_053193710.1;WP_080996608.1;WP_053193713.1;WP_053193714.1;WP_080686176.1;WP_053193720.1;WP_024776307.1;WP_053193723.1;WP_024776309.1;WP_053193727.1;WP_080996609.1;WP_024776312.1;WP_024776313.1

Appendix I - Antismash Cluster organization of secondary metabolites for NZ_LT629798.1

Gene cluster type	Gene cluster genes	Gene cluster gene accessions
	U14_RS11210;BLU14_RS11215	
CLUSTER 5 bacteriocin	BLU14_RS15950;BLU14_RS15955;BLU14_RS15960;BLU14_RS15965;BLU14_RS15970;BLU14_RS15975;BLU14_RS15980;BLU14_RS15985;BLU14_RS15990;BLU14_RS15995;BLU14_RS16000;BLU14_RS16005;BLU14_RS16010	WP_024776369.1;WP_024776368.1;WP_024776367.1;WP_024776366.1;WP_024776365.1;WP_024776364.1;WP_053192802.1;WP_053192800.1;WP_053192798.1;WP_053192797.1;WP_053192796.1;WP_053192869.1;WP_053192795.1
CLUSTER 6 arylpolyene	BLU14_RS20015;BLU14_RS20020;BLU14_RS20025;BLU14_RS20030;BLU14_RS20035;BLU14_RS20040;BLU14_RS20045;BLU14_RS20050;BLU14_RS20055;BLU14_RS20060;BLU14_RS20065;BLU14_RS20070;BLU14_RS20075;BLU14_RS20080;BLU14_RS20085;BLU14_RS20090;BLU14_RS20095;BLU14_RS20100;BLU14_RS20105;BLU14_RS20110;BLU14_RS20115;BLU14_RS20120;BLU14_RS20125;BLU14_RS20130;BLU14_RS20135;BLU14_RS20140;BLU14_RS20145;BLU14_RS20150;BLU14_RS20155;BLU14_RS20160;BLU14_RS20165;BLU14_RS20170;BLU14_RS20175;BLU14_RS20180;BLU14_RS20185;BLU14_RS20190;BLU14_RS20195;BLU14_RS20200;BLU14_RS20205;BLU14_RS20210;BLU14_RS20215	WP_024779858.1;WP_053193400.1;WP_024779860.1;WP_053193236.1;WP_053193234.1;WP_053193232.1;WP_024779864.1;WP_024779865.1;WP_024779866.1;WP_024779867.1;WP_053193229.1;WP_053193226.1;WP_024779870.1;WP_053193223.1;WP_024779872.1;WP_053193219.1;WP_053193216.1;WP_024779875.1;WP_024779876.1;WP_053193214.1;WP_024779878.1;WP_053193211.1;WP_024779880.1;WP_033034765.1;WP_024779883.1;WP_053193208.1;WP_024779885.1;WP_024779886.1;WP_024779887.1;WP_053193205.1;WP_024779889.1;WP_024779890.1;WP_024779891.1;WP_024779892.1;WP_024779893.1;WP_024779894.1;WP_024779895.1;WP_053193397.1;WP_024779897.1;WP_024779898.1;WP_024779899.1

Appendix I - Antismash Cluster organization of secondary metabolites for NZ_LT629798.1

Gene cluster type	Gene cluster genes	Gene cluster gene accessions
CLUSTER 7 other	BLU14_RS21290;BLU14_RS21295;BLU14_RS21300;BLU14_RS21305;BLU14_RS21310;BLU14_RS21315;BLU14_RS21320;BLU14_RS21325;BLU14_RS21330;BLU14_RS21335;BLU14_RS21340;BLU14_RS21345;BLU14_RS21350;BLU14_RS21355;BLU14_RS21360;BLU14_RS21365;BLU14_RS21370;BLU14_RS21375;BLU14_RS21380;BLU14_RS21385;BLU14_RS21390;BLU14_RS21395;BLU14_RS21400;BLU14_RS21405;BLU14_RS21410;BLU14_RS21415;BLU14_RS21420;BLU14_RS21425;BLU14_RS21430	WP_024777922.1;WP_081357984.1;WP_024780723.1;BLU14_RS21305;BLU14_RS21310;WP_053190498.1;WP_024780880.1;WP_024780879.1;WP_053190496.1;WP_053190494.1;WP_053190492.1;WP_024780875.1;WP_053190491.1;WP_024780873.1;WP_024780872.1;WP_024780871.1;WP_053190489.1;WP_024780869.1;WP_053190487.1;WP_024780867.1;WP_024780866.1;WP_024780865.1;WP_024780864.1;WP_053190485.1;WP_053190482.1;WP_024780861.1;WP_024780860.1;WP_053190481.1;WP_024780858.1

Appendix II. Antismash ClusterBlast scores for NZ_LT629798.1 cluster I

Table of genes, locations, strands and annotations of query cluster:				
WP_024776975.1	1502599	1504336	-	dihydroxy-acid dehydratase
WP_024776974.1	1504529	1505852	+	MFS transporter
WP_024776973.1	1506009	1507002	+	fumarylacetoacetate hydrolase
WP_024776972.1	1507015	1508596	+	aldehyde dehydrogenase (NADP(+))
WP_053191113.1	1508894	1514480	-	mannuronan epimerase
WP_024776970.1	1514860	1515304	+	membrane protein
WP_050593780.1	1515357	1516707	+	coproporphyrinogen III oxidase
WP_053191115.1	1516703	1518092	+	oxygen-independent coproporphyrinogen III oxidase
WP_024776967.1	1518268	1518475	-	hypothetical protein
WP_024776966.1	1518481	1519906	-	adeC/adeK/oprM family multidrug efflux complex outer membrane factor
WP_024776965.1	1519993	1520821	-	LuxR family transcriptional regulator
WP_024776964.1	1521313	1522501	-	2-polyprenyl-6-methoxyphenol hydroxylase
WP_053191118.1	1522711	1529977	-	non-ribosomal peptide synthetase
WP_053191119.1	1530071	1531334	-	Glycosyltransferase
WP_053191120.1	1532438	1546022	-	non-ribosomal peptide synthetase
WP_053191121.1	1546025	1562153	-	non-ribosomal peptide synthetase
WP_024780237.1	1562515	1563520	+	transcription factor
WP_024780236.1	1563516	1564887	+	class III aminotransferase
WP_024780235.1	1565004	1565616	+	aspartyl beta-hydroxylase
WP_024780234.1	1565780	1566575	-	AraC family transcriptional regulator
WP_024780233.1	1566701	1566890	+	hypothetical protein
WP_053191123.1	1566900	1567809	+	HlyD family secretion protein
WP_053191125.1	1567826	1569752	+	FUSC family protein
WP_053191127.1	1569772	1569970	+	hypothetical protein

Appendix II. Antismash ClusterBlast scores for NZ_LT629798.1 cluster I

Table of genes, locations, strands and annotations of query cluster:				
WP_041024143.1	1570089	1570296	+	hypothetical protein
WP_024780228.1	1570298	1571078	+	type I methionyl aminopeptidase
WP_053191133.1	1571164	1572790	-	methyl-accepting chemotaxis protein
WP_024780226.1	1573404	1574361	-	EamA/RhaT family transporter
WP_024780225.1	1574357	1574930	-	TetR/AcrR family transcriptional regulator
WP_053191135.1	1575220	1576090	-	hypothetical protein
WP_053191137.1	1576086	1576863	-	alpha/beta hydrolase
WP_053191139.1	1576881	1577871	-	nitronate monooxygenase
WP_024780221.1	1577932	1578301	-	antibiotic biosynthesis monooxygenase
WP_024780220.1	1578329	1578890	-	flavin reductase
WP_024780219.1	1578886	1580056	-	LLM class flavin-dependent oxidoreductase
WP_053191140.1	1580096	1580804	-	dimethylmenaquinone methyltransferase
WP_053191142.1	1580800	1581991	-	isocitrate/isopropylmalate dehydrogenase family protein
WP_053191143.1	1581990	1583013	-	nucleoid-structuring protein H-NS
WP_024780215.1	1583334	1584255	-	LysR family transcriptional regulator
WP_053191145.1	1584345	1585536	-	MFS transporter
WP_053191147.1	1585532	1586534	-	NADPH:quinone reductase
WP_053191150.1	1586612	1589786	-	multidrug efflux RND transporter permease subunit
WP_024780211.1	1589785	1590952	-	MexE family multidrug efflux RND transporter periplasmic adaptor subunit
WP_053191152.1	1590980	1592129	-	Acyltransferase
WP_053191154.1	1592308	1593490	-	hypothetical protein
WP_024780208.1	1593620	1594499	+	LysR family transcriptional regulator
WP_024780207.1	1594514	1595438	-	AraC family transcriptional regulator
WP_024780206.1	1595546	1595888	+	carboxymuconolactone decarboxylase family protein

Appendix II. Antismash ClusterBlast scores for NZ_LT629798.1 cluster I

Table of genes, locations, strands and annotations of query cluster:				
WP_024780709.1	1597089	1598301	-	alpha/beta hydrolase
WP_024780708.1	1598360	1599296	-	chlorinating enzyme
WP_053191156.1	1599313	1601158	-	thioester reductase
WP_024780706.1	1601505	1602567	+	TauD/TfdA family dioxygenase
WP_053191158.1	1602601	1604299	+	ATP-binding protein
BLU14_RS07355	1604609	1630418	+	non-ribosomal peptide synthetase
WP_053193696.1	1633657	1646815	+	non-ribosomal peptide synthetase
WP_081357976.1	1647945	1678842	+	hypothetical protein
WP_024779109.1	1678920	1680072	+	macrolide transporter subunit MacA
WP_053191162.1	1680074	1682036	+	MacB family efflux pump subunit
WP_053191164.1	1682328	1683669	-	2,4-diaminobutyrate 4-aminotransferase
WP_024779112.1	1684009	1685437	+	membrane protein
WP_080996558.1	1685379	1686540	+	efflux RND transporter periplasmic adaptor subunit
WP_053191166.1	1686536	1689620	+	AcrB/AcrD/AcrF family protein
WP_024779115.1	1689677	1690544	-	LuxR family transcriptional regulator
WP_024779116.1	1690576	1691350	-	hypothetical protein
WP_053191168.1	1691939	1692539	+	threonine transporter RhtB
WP_024779118.1	1692625	1693354	-	LuxR family transcriptional regulator
WP_053191170.1	1693707	1696365	-	excinuclease ABC subunit UvrA
WP_053191173.1	1696552	1698031	+	hypothetical protein
WP_053191175.1	1698201	1700376	+	regulatory protein NosR
WP_053191176.1	1700428	1702348	+	nitrous-oxide reductase, TAT-dependent
WP_053191177.1	1702374	1703694	+	copper-binding protein
WP_053191179.1	1703690	1704608	+	ABC transporter ATP-binding protein

Appendix II. Antismash ClusterBlast scores for NZ_LT629798.1 cluster I

Table of genes, locations, strands and annotations of query cluster:				
WP_053191182.1	1704604	1705435	+	hypothetical protein
WP_053191183.1	1705466	1705994	+	NosL protein
WP_053191185.1	1706075	1707572	-	cytochrome C oxidase Cbb3
WP_053191187.1	1707568	1708408	-	uroporphyrinogen-III C-methyltransferase
WP_024779129.1	1708433	1709615	-	heme d1 biosynthesis radical SAM protein NirJ
WP_024779130.1	1709767	1710301	-	Lrp/AsnC family transcriptional regulator
WP_024779131.1	1710275	1710719	-	Lrp/AsnC family transcriptional regulator
WP_053191189.1	1710711	1711224	-	Lrp/AsnC family transcriptional regulator
WP_053191191.1	1711220	1711679	-	Lrp/AsnC family transcriptional regulator
Significant hits:				
Details:				

Appendix III. Transcripts quantification in GL2 mutants of genes differentially expressed in both GL2 and GLRFIA

ID11	Contig	LogCPM	LogFC	P value	Gene product	Diff. expr.	GO
oprM_3	PCO_124	6.80	-2.32	3,18E-34	Outer membrane protein OprM	over GL2	transporter activity
bepE_1	PCO_124	7.91	-2.06	2,24E-19	Efflux pump membrane transporter BepE	over GL2	transporter activity
fct_2	PCO_121	7.88	-1.71	2,40E-03	Ferrichrysobactin receptor	over GL2	transporter activity
HI_0362	PCO_118	8.64	-1.57	7,67E-04	putative periplasmic iron-binding protein	over GL2	membrane protein
ahpF	PCO_123	10.76	-1.40	1,16E-05	Alkyl hydroperoxide reductase subunit F	over GL2	Redox and Oxidative stress
trxB_3	PCO_119	6.26	-1.35	5,04E-09	Thioredoxin reductase	over GL2	Redox and Oxidative stress
bepE_2	PCO_35	10.33	-1.32	8,19E-06	Efflux pump membrane transporter BepE	over GL2	transporter activity
mdtE	PCO_124	7.68	-1.30	1,88E-08	Multidrug resistance protein MdtE	over GL2	transporter activity
ttgI	PCO_35	9.38	-1.26	3,56E-05	Toluene efflux pump outer membrane protein TtgI	over GL2	transporter activity
bepF	PCO_35	10.42	-1.21	4,62E-05	Efflux pump periplasmic linker BepF	over GL2	transporter activity
cat_1	PCO_114	11.24	-1.09	1,23E-02	Catalase	over GL2	transporter activity
katB	PCO_81	9.08	-1.07	1,36E-03	Catalase	over GL2	Redox and Oxidative stress
ahpC	PCO_123	11.88	-1.01	2,52E-03	Alkyl hydroperoxide reductase subunit C	over GL2	Redox and Oxidative stress
Alvin_1094	PCO_81	6.34	-0.99	4,79E-06	hypothetical protein	over GL2	unknown
yceJ_1	PCO_109	6.89	-0.92	1,32E-04	hypothetical protein	over GL2	unknown
qorA	PCO_112	10.15	-0.91	7,93E-03	Quinone oxidoreductase 1	over GL2	Redox and Oxidative stress
ydfG_1	PCO_142	5.47	-0.86	1,05E-04	putative protein YdfG	over GL2	unknown
mmgC_2	PCO_101	11.01	-0.82	3,51E-02	Acyl-CoA dehydrogenase	over GL2	fatty acid metabolism
fhaB_1	PCO_106	7.90	-0.81	7,84E-03	Filamentous hemagglutinin	over GL2	unknown
gutR	PCO_113	9.30	-0.80	2,86E-02	Transcription activator GutR	over GL2	regulation of transcription.
eamB_3	PCO_127	5.81	-0.79	1,32E-04	Cysteine/O-acetylserine efflux protein	over GL2	transporter activity
ydeR	PCO_71	7.35	-0.78	7,84E-03	putative MFS-type transporter YdeR	over GL2	unknown
alr_2	PCO_72	7.38	-0.77	2,79E-02	Alanine racemase	over GL2	aminoacid metabolism
PA3287	PCO_71	7.26	-0.74	1,58E-02	Putative ankyrin repeat protein	over GL2	unknown
yeiR	PCO_118	6.75	-0.74	5,61E-03	putative protein YeiR	over GL2	others
tcmH	PCO_127	5.27	-0.72	4,79E-02	Tetracenomycin-F1 monooxygenase	over GL2	Secondary metabolite production

Appendix III. Transcripts quantification in GL2 mutants of genes differentially expressed in both GL2 and GLRFIA

ID11	Contig	LogCPM	LogFC	P value	Gene product	Diff. expr.	GO
hyuE	PCO_127	3.92	-0.71	5,10E-03	Hydantoin racemase	over GL2	aminoacid metabolism
ywnA	PCO_119	5.28	-0.71	9,51E-04	Putative HTH-type transcriptional regulator YwnA	over GL2	regulation of transcription.
fabR	PCO_103	7.10	-0.64	3,51E-02	HTH-type transcriptional repressor FabR	over GL2	regulation of transcription.
DDB_G0269096	PCO_153	4.85	0.55	3,61E-02	Transmembrane protein	over WT	membrane protein
FI	PCO_115	4.73	0.58	2,45E-02	Major tail sheath protein	over WT	others
ydcS	PCO_108	6.63	0.63	2,63E-02	Putative ABC transporter periplasmic-binding protein YdcS	over WT	transporter activity
prr_2	PCO_108	6.29	0.69	3,71E-03	Gamma-aminobutyraldehyde dehydrogenase	over WT	Redox and Oxidative stress
N	PCO_115	4.97	0.70	8,67E-03	Capsid proteins	over WT	unknown
SSU1	PCO_96	6.26	0.74	1,90E-02	Sulfite efflux pump SSU1	over WT	transporter activity
RP587_1	PCO_124	5.60	0.74	4,74E-02	SCO2-like protein RP587	over WT	transporter activity
cyc1	PCO_124	5.10	0.84	8,25E-04	Cytochrome c-552	over WT	Redox and Oxidative stress
preA	PCO_90	7.27	0.93	1,80E-03	NAD-dependent dihydropyrimidine dehydrogenase subunit PreA	over WT	Purine and pirimidine metabolism
Rv2030c_2	PCO_112	6.33	0.96	1,09E-03	putative proteinc/MT2089	over WT	unknown
fabG_2	PCO_124	6.11	1.00	1,79E-03	3-oxoacyl-[acyl-carrier-protein] reductase FabG	over WT	fatty acid metabolism
nemA_2	PCO_124	6.79	1.03	6,12E-04	N-ethylmaleimide reductase	over WT	Redox and Oxidative stress
nirC	PCO_105	4.99	1.08	1,11E-03	Cytochrome c55X	over WT	Redox and Oxidative stress
MW2112	PCO_107	5.54	1.09	1,05E-04	Zinc-type alcohol dehydrogenase-like protein	over WT	Redox and Oxidative stress
NGR_a01370_2	PCO_124	6.23	1.12	6,09E-05	Putative aldehyde-dehydrogenase-like protein y4uC	over WT	fatty acid metabolism
aq_1546	PCO_124	5.34	1.16	3,03E-04	putative phosphosugar isomerase	over WT	carbohydrate metabolic process
pyd1	PCO_90	7.58	1.19	2,73E-05	Dihydropyrimidine dehydrogenase [NADP(+)]	over WT	Purine and pirimidine metabolism
dht	PCO_90	8.90	1.34	3,16E-05	D-hydantoinase/dihydropyrimidinase	over WT	Purine and pirimidine

Appendix III. Transcripts quantification in GL2 mutants of genes differentially expressed in both GL2 and GLRFIA

ID11	Contig	LogCPM	LogFC	P value	Gene product	Diff. expr.	GO
							metabolism
ybfB_1	PCO_124	6.89	1.35	1,38E-06	putative MFS-type transporter YbfB	over WT	others
pbuE	PCO_115	6.74	1.37	2,41E-06	Purine efflux pump PbuE	over WT	transporter activity
cdhR_9	PCO_124	7.52	1.51	2,41E-06	HTH-type transcriptional regulator CdhR	over WT	regulation of transcription.
sstT	PCO_115	6.89	1.55	9,82E-08	Serine/threonine transporter SstT	over WT	transporter activity
spvB_2	PCO_36	7.78	1.79	5,98E-09	Mono(ADP-ribosyl)transferase SpvB	over WT	Redox and Oxidative stress
algG	PCO_120	7.48	1.93	2,31E-04	Poly(beta-D-mannuronate) C5 epimerase	over WT	alginic acid biosynthetic process
algE	PCO_120	7.56	1.95	1,80E-03	Alginate production protein AlgE	over WT	alginic acid biosynthetic process
algI_1	PCO_120	6.71	2.27	8,00E-06	putative alginate O-acetylase AlgI	over WT	alginic acid biosynthetic process
algJ_1	PCO_120	6.78	2.28	1,23E-06	putative alginate O-acetylase AlgJ	over WT	alginic acid biosynthetic process
algL	PCO_120	7.07	2.29	2,20E-05	Alginate lyase	over WT	alginic acid biosynthetic process
algX	PCO_120	7.13	2.33	4,59E-05	Alginate biosynthesis protein AlgX	over WT	alginic acid biosynthetic process
nolG_1	PCO_105	7.42	2.50	1,44E-18	Nodulation protein NolG	over WT	transporter activity
algK	PCO_120	6.87	2.64	1,70E-05	Alginate biosynthesis protein AlgK	over WT	alginic acid biosynthetic process
alg8	PCO_120	7.44	2.70	9,07E-05	Glycosyltransferase alg8	over WT	alginic acid biosynthetic process
dltE_2	PCO_124	6.86	2.85	1,93E-20	putative oxidoreductase DltE	over WT	Redox and Oxidative stress
yeaM_3	PCO_85	7.12	3.05	1,67E-16	putative HTH-type transcriptional regulator YeaM	over WT	regulation of transcription.
algA	PCO_120	9.87	3.05	1,83E-06	Alginate biosynthesis protein AlgA	over WT	alginic acid biosynthetic process

Appendix III. Transcripts quantification in GL2 mutants of genes differentially expressed in both GL2 and GLRFIA

ID11	Contig	LogCPM	LogFC	P value	Gene product	Diff. expr.	GO
arpC	PCO_126	9.01	3.07	2,05E-11	Antibiotic efflux pump outer membrane protein ArpC	over WT	membrane protein
alg44	PCO_120	7.16	3.09	2,28E-05	Alginate biosynthesis protein Alg44	over WT	alginic acid biosynthetic process
gph_2	PCO_88	6.25	3.15	3,81E-18	Phosphoglycolate phosphatase	over WT	carbohydrate metabolic process
DIT1_1	PCO_88	8.27	3.21	1,94E-15	Spore wall maturation protein DIT1	over WT	unknown
DIT1_2	PCO_88	8.09	3.30	9,66E-14	Spore wall maturation protein DIT1	over WT	unknown
algF	PCO_120	7.89	3.38	9,41E-09	Alginate biosynthesis protein AlgF	over WT	alginic acid biosynthetic process
mefA	PCO_85	6.94	3.49	2,11E-14	Macrolide efflux protein A	over WT	transporter activity
nccB	PCO_105	5.77	3.52	3,13E-31	Nickel-cobalt-cadmium resistance protein NccB	over WT	stress response
Hif1an	PCO_85	7.26	3.95	1,99E-13	Hypoxia-inducible factor 1-alpha inhibitor	over WT	Redox and Oxidative stress
azoB_4	PCO_124	8.50	4.05	1,93E-24	NAD(P)H azoreductase	over WT	Redox and Oxidative stress
algD	PCO_120	10.12	4.09	2,50E-06	GDP-mannose 6-dehydrogenase	over WT	alginic acid biosynthetic process
oprM_1	PCO_105	6.90	4.39	2,27E-29	Outer membrane protein OprM	over WT	transporter activity
rhbA_1	PCO_105	7.94	4.42	2,22E-18	Diaminobutyrate--2-oxoglutarate aminotransferase	over WT	Secondary metabolite production
hpxO	PCO_126	8.74	4.63	2,14E-26	FAD-dependent urate hydroxylase	over WT	Purine and pirimidine metabolism
macB2	PCO_105	9.30	4.71	7,55E-33	Macrolide export ATP-binding/permease protein MacB 2	over WT	transporter activity
dhbF_1	PCO_126	10.80	4.85	1,91E-29	Dimodular nonribosomal peptide synthase	over WT	Secondary metabolite production
pcaD_2	PCO_64	9.53	4.89	5,40E-18	3-oxoadipate enol-lactonase 1	over WT	Secondary metabolite production
yddQ_1	PCO_114	9.61	4.92	2,79E-09	putative isochorismatase family protein YddQ	over WT	Others

Appendix III. Transcripts quantification in GL2 mutants of genes differentially expressed in both GL2 and GLRFIA

ID11	Contig	LogCPM	LogFC	P value	Gene product	Diff. expr.	GO
dhbF_4	PCO_83	7.74	5.19	2,75E-58	Dimodular nonribosomal peptide synthase	over WT	Secondary metabolite production
syrD_2	PCO_64	9.36	5.28	5,50E-18	ATP-binding protein SyrD	over WT	Secondary metabolite production
tycB	PCO_1	6.62	5.30	2,14E-66	Tyrocidine synthase 2	over WT	Secondary metabolite production
ATG26	PCO_126	9.05	5.72	5,29E-21	Sterol 3-beta-glucosyltransferase	over WT	transporter activity
grsB_1	PCO_78	11.53	5.76	1,34E-47	Gramicidin S synthase 2	over WT	Secondary metabolite production
ppsE_1	PCO_105	10.89	5.78	9,82E-59	Plipastatin synthase subunit E	over WT	Secondary metabolite production
macA	PCO_105	8.96	5.85	2,47E-45	Macrolide export protein MacA	over WT	transporter activity
grsB_2	PCO_80	11.71	6.08	5,52E-54	Gramicidin S synthase 2	over WT	Secondary metabolite production
At3g21360_3	PCO_64	9.12	6.15	2,34E-18	Clavaminate synthase-like protein	over WT	Redox and Oxidative stress
dhbF_3	PCO_64	11.24	6.99	6,28E-28	Dimodular nonribosomal peptide synthase	over WT	Secondary metabolite production

Appendix IV. Transcripts quantification in GLRFIA mutants of genes differentially expressed in both GL2 and GLRFIA							
ID	**Contig**	**LogCPM**	**LogFC**	**P value**	**Gene product**	**Diff expres**	**GO**
oprM_3	PCO_124	6,83	-2,13	0,00060	Outer membrane protein OprM	over GLRFIA	transporter activity
bepE_1	PCO_124	8,05	-2,03	0,00082	Efflux pump membrane transporter BepE	over GLRFIA	transporter activity
ahpF	PCO_123	11,22	-1,85	0,00000	Alkyl hydroperoxide reductase subunit F	over GLRFIA	Redox and Oxidative stress
cat_1	PCO_114	11,72	-1,63	0,00000	Catalase	over GLRFIA	transporter activity
mdtE	PCO_124	8,01	-1,61	0,00000	Multidrug resistance protein MdtE	over GLRFIA	transporter activity
fct_2	PCO_121	7,83	-1,46	0,03545	Ferrichrysobactin receptor	over GLRFIA	transporter activity
HI_0362	PCO_118	8,65	-1,40	0,01480	putative periplasmic iron-binding protein	over GLRFIA	membrane protein
ahpC	PCO_123	12,18	-1,33	0,00000	Alkyl hydroperoxide reductase subunit C	over GLRFIA	Redox and Oxidative stress
trxB_3	PCO_119	6,34	-1,30	0,00225	Thioredoxin reductase	over GLRFIA	Redox and Oxidative stress
katB	PCO_81	9,24	-1,18	0,00045	Catalase	over GLRFIA	Redox and Oxidative stress
Alvin_1094	PCO_81	6,50	-1,12	0,00083	hypothetical protein	over GLRFIA	unknown
bepF	PCO_35	10,45	-1,12	0,00010	Efflux pump periplasmic linker BepF	over GLRFIA	transporter activity
bepE_2	PCO_35	10,26	-1,06	0,00366	Efflux pump membrane transporter BepE	over GLRFIA	transporter activity
PA3287	PCO_71	7,51	-1,04	0,00085	Putative ankyrin repeat protein	over GLRFIA	unknown
ttgI	PCO_35	9,32	-1,02	0,00528	Toluene efflux pump outer membrane protein TtgI	over GLRFIA	transporter activity
eamB_3	PCO_127	5,98	-0,97	0,00000	Cysteine/O-acetylserine efflux protein	over GLRFIA	transporter activity
mmgC_2	PCO_101	11,16	-0,96	0,00298	Acyl-CoA dehydrogenase	over GLRFIA	fatty acid metabolism

Appendix IV. Transcripts quantification in GLRFIA mutants of genes differentially expressed in both GL2 and GLRFIA							
ID	**Contig**	**LogCPM**	**LogFC**	**P value**	**Gene product**	**Diff expres**	**GO**
qorA	PCO_112	10,25	-0,95	0,00221	Quinone oxidoreductase 1	over GLRFIA	Redox and Oxidative stress
alr_2	PCO_72	7,55	-0,94	0,00543	Alanine racemase	over GLRFIA	transporter activity
tcmH	PCO_127	5,42	-0,88	0,00554	Tetracenomycin-F1 monooxygenase	over GLRFIA	Secondary metabolite production
gutR	PCO_113	9,35	-0,80	0,03499	Transcription activator GutR	over GLRFIA	regulation of transcription.
yceJ_1	PCO_109	6,88	-0,79	0,00187	hypothetical protein	over GLRFIA	unknown
fhaB_1	PCO_106	7,96	-0,79	0,04930	Filamentous hemagglutinin	over GLRFIA	unknown
ydeR	PCO_71	7,41	-0,77	0,00586	putative MFS-type transporter YdeR	over GLRFIA	transporter activity
fabR	PCO_103	7,21	-0,74	0,02078	HTH-type transcriptional repressor FabR	over GLRFIA	regulation of transcription.
ywnA	PCO_119	5,36	-0,74	0,00278	Putative HTH-type transcriptional regulator YwnA	over GLRFIA	regulation of transcription.
hyuE	PCO_127	3,99	-0,74	0,02595	Hydantoin racemase	over GLRFIA	aminoacid metabolism
yeiR	PCO_118	6,77	-0,69	0,01573	putative protein YeiR	over GLRFIA	Others
ydfG_1	PCO_142	5,36	-0,58	0,03696	putative protein YdfG	over GLRFIA	Others
prr_2	PCO_108	6,24	0,59	0,03424	Gamma-aminobutyraldehyde dehydrogenase	over WT	Redox and Oxidative stress
ydcS	PCO_108	6,57	0,59	0,04552	Putative ABC transporter periplasmic-binding protein YdcS	over WT	transporter activity
DDB_G0269096	PCO_153	4,73	0,68	0,00271	Transmembrane protein	over WT	membrane protein
fabG_2	PCO_124	6,10	0,72	0,03424	3-oxoacyl-[acyl-carrier-protein] reductase FabG	over WT	fatty acid metabolism metabolism
cyc1	PCO_124	5,04	0,72	0,00199	Cytochrome c-552	over WT	Redox and Oxidative stress

Appendix IV. Transcripts quantification in GLRFIA mutants of genes differentially expressed in both GL2 and GLRFIA							
ID	**Contig**	**LogCPM**	**LogFC**	**P value**	**Gene product**	**Diff expres**	**GO**
FI	PCO_115	4,60	0,74	0,00132	Major tail sheath protein	over WT	Others
Rv2030c_2	PCO_112	6,28	0,78	0,03019	putative proteinc/MT2089	over WT	unknown
MW2112	PCO_107	5,52	0,79	0,00333	Zinc-type alcohol dehydrogenase-like protein	over WT	Redox and Oxidative stress
SSU1	PCO_96	6,15	0,79	0,01210	Sulfite efflux pump SSU1	over WT	transporter activity
nirC	PCO_105	4,97	0,80	0,02559	Cytochrome c55X	over WT	Redox and Oxidative stress
N	PCO_115	4,84	0,81	0,00011	Capsid proteins	over WT	unknown
RP587_1	PCO_124	5,44	0,95	0,00045	SCO2-like protein RP587	over WT	transporter activity
spvB_2	PCO_36	7,79	1,11	0,00003	Mono(ADP-ribosyl)transferase SpvB	over WT	Redox and Oxidative stress
nemA_2	PCO_124	6,65	1,11	0,00006	N-ethylmaleimide reductase	over WT	Redox and Oxidative stress
aq_1546	PCO_124	5,21	1,20	0,00001	putative phosphosugar isomerase	over WT	carbohydrate metabolic process
preA	PCO_90	7,07	1,23	0,00000	NAD-dependent dihydropyrimidine dehydrogenase subunit PreA	over WT	Purine and pirimidine metabolism
NGR_a01370_2	PCO_124	6,07	1,26	0,00000	Putative aldehyde-dehydrogenase-like protein y4uC	over WT	fatty acid metabolism
pbuE	PCO_115	6,62	1,29	0,00000	Purine efflux pump PbuE	over WT	transporter activity
Dht	PCO_90	8,78	1,29	0,00002	D-hydantoinase/dihydropyrimidinase	over WT	Purine and pirimidine metabolism
ybfB_1	PCO_124	6,74	1,37	0,00000	putative MFS-type transporter YbfB	over WT	Others
cdhR_9	PCO_124	7,39	1,47	0,00000	HTH-type transcriptional regulator CdhR	over WT	regulation of transcription.
pyd1	PCO_90	7,37	1,49	0,00000	Dihydropyrimidine dehydrogenase [NADP(+)]	over WT	Purine and pirimidine metabolism
sstT	PCO_115	6,74	1,51	0,00000	Serine/threonine transporter SstT	over WT	transporter activity

Appendix IV. Transcripts quantification in GLRFIA mutants of genes differentially expressed in both GL2 and GLRFIA							
ID	**Contig**	**LogCPM**	**LogFC**	**P value**	**Gene product**	**Diff expres**	**GO**
algG	PCO_120	7,28	2,00	0,00000	Poly(beta-D-mannuronate) C5 epimerase	over WT	alginic acid biosynthetic process
algI_1	PCO_120	6,53	2,15	0,00000	putative alginate O-acetylase AlgI	over WT	alginic acid biosynthetic process
algE	PCO_120	7,33	2,22	0,00000	Alginate production protein AlgE	over WT	alginic acid biosynthetic process
algX	PCO_120	6,91	2,42	0,00000	Alginate biosynthesis protein AlgX	over WT	alginic acid biosynthetic process
algJ_1	PCO_120	6,55	2,43	0,00000	putative alginate O-acetylase AlgJ	over WT	alginic acid biosynthetic process
algL	PCO_120	6,84	2,45	0,00000	Alginate lyase	over WT	alginic acid biosynthetic process
algK	PCO_120	6,66	2,56	0,00000	Alginate biosynthesis protein AlgK	over WT	alginic acid biosynthetic process
nolG_1	PCO_105	7,19	2,59	0,00000	Nodulation protein NolG	over WT	transporter activity
dltE_2	PCO_124	6,66	2,71	0,00000	putative oxidoreductase DltE	over WT	Redox and Oxidative stress
alg8	PCO_120	7,21	2,75	0,00000	Glycosyltransferase alg8	over WT	alginic acid biosynthetic process
arpC	PCO_126	8,79	2,92	0,00000	Antibiotic efflux pump outer membrane protein ArpC	over WT	membrane protein
yeaM_3	PCO_85	6,90	2,93	0,00000	putative HTH-type transcriptional regulator YeaM	over WT	regulation of transcription.
algA	PCO_120	9,65	2,98	0,00000	Alginate biosynthesis protein AlgA	over WT	alginic acid biosynthetic process
algF	PCO_120	7,65	3,28	0,00000	Alginate biosynthesis protein AlgF	over WT	alginic acid biosynthetic process
alg44	PCO_120	6,90	3,30	0,00000	Alginate biosynthesis protein Alg44	over WT	alginic acid

Appendix IV. Transcripts quantification in GLRFIA mutants of genes differentially expressed in both GL2 and GLRFIA							
ID	**Contig**	**LogCPM**	**LogFC**	**P value**	**Gene product**	**Diff expres**	**GO**
							biosynthetic process
gph_2	PCO_88	5,99	3,34	0,00000	Phosphoglycolate phosphatase	over WT	carbohydrate metabolic process
DIT1_2	PCO_88	7,83	3,52	0,00000	Spore wall maturation protein DIT1	over WT	unknown
DIT1_1	PCO_88	8,00	3,57	0,00000	Spore wall maturation protein DIT1	over WT	unknown
mefA	PCO_85	6,67	3,68	0,00000	Macrolide efflux protein A	over WT	transporter activity
nccB	PCO_105	5,51	3,83	0,00000	Nickel-cobalt-cadmium resistance protein NccB	over WT	stress response
Hif1an	PCO_85	7,00	4,01	0,00000	Hypoxia-inducible factor 1-alpha inhibitor	over WT	Redox and Oxidative stress
algD	PCO_120	9,86	4,09	0,00000	GDP-mannose 6-dehydrogenase	over WT	alginic acid biosynthetic process
azoB_4	PCO_124	8,24	4,23	0,00000	NAD(P)H azoreductase	over WT	Redox and Oxidative stress
oprM_1	PCO_105	6,64	4,29	0,00000	Outer membrane protein OprM	over WT	transporter activity
hpxO	PCO_126	8,47	4,69	0,00000	FAD-dependent urate hydroxylase	over WT	Purine and pirimidine metabolism
rhbA_1	PCO_105	7,66	4,81	0,00000	Diaminobutyrate--2-oxoglutarate aminotransferase	over WT	Secondary metabolite 1production
macB2	PCO_105	9,03	4,83	0,00000	Macrolide export ATP-binding/permease protein MacB 2	over WT	transporter activity
dhbF_1	PCO_126	10,53	4,84	0,00000	Dimodular nonribosomal peptide synthase	over WT	Secondary metabolite production
pcaD_2	PCO_64	9,26	4,94	0,00000	3-oxoadipate enol-lactonase 1	over WT	Secondary metabolite production
dhbF_4	PCO_83	7,48	5,02	0,00000	Dimodular nonribosomal peptide synthase	over WT	Secondary metabolite production
yddQ_1	PCO_114	9,33	5,09	0,00000	putative isochorismatase family	over WT	Others

Appendix IV. Transcripts quantification in GLRFIA mutants of genes differentially expressed in both GL2 and GLRFIA							
ID	**Contig**	**LogCPM**	**LogFC**	**P value**	**Gene product**	**Diff expres**	**GO**
					protein YddQ		
tycB	PCO_1	6,34	5,42	0,00000	Tyrocidine synthase 2	over WT	Secondary metabolite production
syrD_2	PCO_64	9,08	5,51	0,00000	ATP-binding protein SyrD	over WT	Secondary metabolite production
macA	PCO_105	8,68	5,69	0,00000	Macrolide export protein MacA	over WT	transporter activity
ATG26	PCO_126	8,77	5,72	0,00000	Sterol 3-beta-glucosyltransferase	over WT	transporter activity
ppsE_1	PCO_105	10,61	5,79	0,00000	Plipastatin synthase subunit E	over WT	Secondary metabolite production
grsB_1	PCO_78	11,25	5,95	0,00000	Gramicidin S synthase 2	over WT	Secondary metabolite production
grsB_2	PCO_80	11,43	6,25	0,00000	Gramicidin S synthase 2	over WT	Secondary metabolite production
At3g21360_3	PCO_64	8,83	6,55	0,00000	Clavaminate synthase-like protein	over WT	Redox and Oxidative stress
dhbF_3	PCO_64	10,95	7,18	0,00000	Dimodular nonribosomal peptide synthase	over WT	Secondary metabolite production

Appendix V. Transcripts quantification genes differentially expressed only in GL2							
ID	**contig**	**LogCPM**	**LogFC**	**PVALUE**	**gene_product**	**Diff.expr.**	**GO**
HI_1624	PCO_121	7.92	-2.06	0,01722	putative protein	over GL2	unknown
TM_0124	PCO_118	8.12	-1.94	0,02598	putative metal transport system ATP-binding protein	over GL2	transporter activity
folE2_1	PCO_118	9.15	-1.91	0,02454	GTP cyclohydrolase FolE2	over GL2	unknown
yjgH	PCO_102	4.12	-1.56	0,00000	RutC family protein YjgH	over GL2	unknown
CBSX3_1	PCO_119	5.26	-1.07	0,02786	CBS domain-containing protein CBSX3%2C mitochondrial	over GL2	unknown
MJ0761	PCO_26	7.74	-0.96	0,00380	putative deoxyribonuclease	over GL2	unknown
yjcH	PCO_71	7.73	-0.94	0,00180	Inner membrane protein YjcH	over GL2	unknown
fusA_3	PCO_70	7.29	-0.90	0,00019	Fusaric acid resistance protein FusA	over GL2	unknown
actP_2	PCO_71	9.02	-0.89	0,01447	Cation/acetate symporter ActP	over GL2	Redox and Oxidative stress
ygaZ	PCO_127	5.06	-0.87	0,00006	Inner membrane protein YgaZ	over GL2	transporter activity
HI_1511	PCO_88	8.68	-0.85	0,03903	Mu-like prophage FluMu tail sheath protein	over GL2	unknown
aroB_1	PCO_26	8.22	-0.74	0,03834	3-dehydroquinate synthase	over GL2	aminoacid metabolism
hpmB_2	PCO_24	5.63	-0.69	0,00308	Hemolysin transporter protein HpmB	over GL2	transporter activity
HI_1008	PCO_110	6.89	-0.67	0,01071	putative protein	over GL2	unknown
plu0081	PCO_154	5.16	-0.67	0,00407	hypothetical protein	over GL2	unknown
ykwD_2	PCO_119	6.20	-0.55	0,02454	putative protein YkwD	over GL2	others
pucI_2	PCO_127	5.39	-0.55	0,02897	putative allantoin permease	over GL2	transporter activity
aaeB	PCO_85	5.28	0.61	0,03512	p-hydroxybenzoic acid efflux pump subunit AaeB	over WT	transporter activity
Acad10	PCO_95	6.57	0.65	0,03512	Acyl-CoA dehydrogenase family member 10	over WT	Redox and Oxidative stress
HMT3	PCO_118	7.31	0.66	0,04219	Homocysteine S-methyltransferase 3	over WT	aminoacid metabolism
osmY_3	PCO_124	6.07	0.67	0,04743	Osmotically-inducible protein Y	over WT	transporter activity
M	PCO_115	3.33	0.68	0,04984	Terminase%2C endonuclease subunit	over WT	others
uspE_1	PCO_109	6.33	0.70	0,04504	hypothetical protein	over WT	stress protein
groL5	PCO_124	6.72	0.72	0,03811	60 kDa chaperonin 5	over WT	stress protein
yhbT	PCO_140	5.50	0.74	0,04701	putative protein YhbT	over WT	transporter activity

Appendix V. Transcripts quantification genes differentially expressed only in GL2							
ID	**contig**	**LogCPM**	**LogFC**	**PVALUE**	**gene_product**	**Diff.expr.**	**GO**
greB_2	PCO_56	5.92	0.77	0,01126	Transcription elongation factor GreB	over WT	regulation of transcription.
uspE_6	PCO_124	5.51	0.79	0,00510	Universal stress protein E	over WT	stress protein
AF_1420	PCO_117	7.68	0.79	0,03696	putative protein	over WT	unknown
pdhC	PCO_112	5.82	0.82	0,01758	Dihydrolipoyllysine-residue acetyltrans component of pyr dehydr complex	over WT	carbohydrate metabolic process
DR_1438	PCO_112	5.22	0.86	0,00509	putative ABC transporter-binding protein	over WT	transporter activity
PA4778	PCO_107	5.24	0.86	0,00041	putative HTH-type transcriptional regulator	over WT	regulation of transcription.
osmY_2	PCO_112	6.90	0.87	0,01369	Osmotically-inducible protein Y	over WT	stress protein
hcnA_3	PCO_90	7.71	0.88	0,04198	Hydrogen cyanide synthase subunit HcnA	over WT	Secondary metabolite production
adhA	PCO_109	6.63	0.88	0,02170	putative alcohol dehydrogenase AdhA	over WT	Redox and Oxidative stress
osmY_1	PCO_109	7.23	0.88	0,01018	Osmotically-inducible protein Y	over WT	transporter activity
Rv0571c_1	PCO_112	5.52	0.89	0,02061	Putative phosphoribosyl transferasec/MT0597	over WT	others
trpI_2	PCO_156	6.72	0.90	0,00022	HTH-type transcriptional regulator TrpI	over WT	regulation of transcription.
yhbU	PCO_140	7.03	0.91	0,03270	putative protease YhbU	over WT	others
cdhR_10	PCO_124	7.41	0.91	0,00856	HTH-type transcriptional regulator CdhR	over WT	regulation of transcription.
uspE_2	PCO_109	7.11	0.92	0,01167	Universal stress protein E	over WT	stress protein
RPA1673	PCO_109	7.98	0.93	0,03040	putative phosphoketolase	over WT	carbohydrate metabolic process
bkdA1	PCO_123	7.81	0.93	0,01976	2-oxoisovalerate dehydrogenase subunit alpha	over WT	Redox and Oxidative stress
yfiQ	PCO_109	7.39	0.94	0,00404	putative protein YfiQ	over WT	unknown
Rv2030c_1	PCO_112	7.56	0.97	0,01758	putative proteinc/MT2089	over WT	unknown
pdxH_1	PCO_140	7.95	0.99	0,03354	Pyridoxine/pyridoxamine 5'-phosphate oxidase	over WT	Redox and Oxidative stress
yhiI	PCO_109	7.32	1.00	0,01758	putative protein YhiI	over WT	transporter activity
uspE_5	PCO_112	7.02	1.03	0,00083	Universal stress protein E	over WT	unknown
hemN_2	PCO_126	9.31	1.05	0,00784	Oxygen-independent coproporphyrinogen-III oxidase	over WT	Redox and Oxidative stress
arcD	PCO_109	8.95	1.05	0,01409	Arginine/ornithine antiporter	over WT	transporter activity

Appendix V. Transcripts quantification genes differentially expressed only in GL2							
ID	**contig**	**LogCPM**	**LogFC**	**PVALUE**	**gene_product**	**Diff.expr.**	**GO**
nirM	PCO_105	7.70	1.05	0,00793	Cytochrome c-551	over WT	Redox and Oxidative stress
pyrR_2	PCO_112	6.20	1.08	0,00252	Bifunctional protein PyrR	over WT	regulation of transcription.
echR	PCO_105	7.28	1.10	0,00013	Transcriptional activator protein EchR	over WT	regulation of transcription.
uspE_4	PCO_109	9.47	1.13	0,01443	Universal stress protein E	over WT	stress protein
bkdA2	PCO_123	6.78	1.19	0,00057	2-oxoisovalerate dehydrogenase subunit beta	over WT	unknow
hemN_3	PCO_126	9.45	1.23	0,00896	Oxygen-independent coproporphyrinogen-III oxidase	over WT	Redox and Oxidative stress
map_2	PCO_85	6.79	1.24	0,00106	Methionine aminopeptidase	over WT	aminoacid metabolism
copA_2	PCO_107	7.82	1.52	0,00000	Copper-exporting P-type ATPase A	over WT	transporter activity
copZ_1	PCO_107	4.88	2.01	0,00000	Copper chaperone CopZ	over WT	transporter activity
copZ_2	PCO_156	6.18	2.27	0,00000	Copper chaperone CopZ	over WT	transporter activity
traI	PCO_105	9.32	5.60	0,00000	Acyl-homoserine-lactone synthase	over WT	quorum sensing

Appendix VI. Transcripts quantification genes differentially expressed only in GLRFIA							
ID	**contig**	**LogCPM**	**LogFC**	**P value**	**Gene_product**	**Diff. Expr.**	**GO**
Cyp4d2	PCO_116	6,92	-2,49	0,04552	Cytochrome P450 4d2	over GLRFIA	Redox and Oxidative
PA4923	PCO_103	7,54	-1,00	0,01002	LOG family protein	over GLRFIA	Others
aq_740	PCO_148	8,88	-0,96	0,00068	hypothetical protein	over GLRFIA	transporter activity
GPX2	PCO_128	7,75	-0,96	0,00119	Glutathione peroxidase 2	over GLRFIA	Redox and Oxidative
yadG	PCO_127	9,41	-0,91	0,00390	putative ABC transporter ATP-binding protein YadG	over GLRFIA	transporter activity
algE3	PCO_112	5,74	-0,86	0,00017	Poly(beta-D-mannuronate) C5 epimerase 3	over GLRFIA	alginic acid biosynthe process
mtcA1	PCO_124	11,24	-0,86	0,00543	Beta-carbonic anhydrase 1	over GLRFIA	Others
ribA	PCO_114	8,36	-0,85	0,00543	GTP cyclohydrolase-2	over GLRFIA	Others
Rv0196	PCO_124	6,73	-0,81	0,00294	putative HTH-type transcriptional regulator/MT0206	over GLRFIA	regulation of transcrip
yadH	PCO_127	8,51	-0,77	0,02479	Inner membrane transport permease YadH	over GLRFIA	membrane protein
ohrR_2	PCO_128	6,46	-0,76	0,00826	Organic hydroperoxide resistance transcriptional regulator	over GLRFIA	regulation of transcrip
HD_0322	PCO_72	7,41	-0,75	0,03696	RutC family protein	over GLRFIA	Purine and pirimidine metabolism
Mb2924c	PCO_117	11,02	-0,72	0,03361	putative oxidoreductasec	over GLRFIA	Redox and Oxidative
fdhD	PCO_117	9,26	-0,72	0,02866	hypothetical protein	over GLRFIA	hypothetical protein
yaiW	PCO_47	6,51	-0,70	0,01488	putative protein YaiW	over GLRFIA	Others
yfdC	PCO_109	6,24	-0,69	0,03361	Inner membrane protein YfdC	over GLRFIA	membrane protein
modB	PCO_123	5,75	-0,68	0,00275	Molybdenum transport system permease protein ModB	over GLRFIA	transporter activity
Mb2599c_2	PCO_103	7,01	-0,65	0,03545	putative proteinc	over GLRFIA	Others
yheV	PCO_113	5,55	-0,63	0,03696	putative protein YheV	over GLRFIA	Others
modC	PCO_123	6,85	-0,63	0,02836	Molybdenum import ATP-binding protein ModC	over GLRFIA	Others
fadA_3	PCO_127	6,15	-0,60	0,03361	Putative acyltransferase	over GLRFIA	others
PA1518	PCO_98	5,36	-0,58	0,03423	5-hydroxyisourate hydrolase	over GLRFIA	Others

Appendix VI. Transcripts quantification genes differentially expressed only in GLRFIA							
ID	**contig**	**LogCPM**	**LogFC**	**P value**	**Gene_product**	**Diff. Expr.**	**GO**
R00369_1	PCO_112	5,20	-0,54	0,04274	putative protein	over GLRFIA	others
prmC_2	PCO_153	5,04	0,60	0,01804	Release factor glutamine methyltransferase	over WT	Others
acoA	PCO_119	3,75	0,65	0,03057	Acetoin:2%2C6-dichlorophenolindophenol oxidoreductase subunit alpha	over WT	Redox and Oxidative
fepC	PCO_103	6,10	0,66	0,03564	Ferric enterobactin transport ATP-binding protein FepC	over WT	transporter activity
lysB	PCO_115	3,05	0,68	0,02595	Protein lysB	over WT	Others
jayE	PCO_88	5,68	0,68	0,04552	Putative protein JayE from lambdoid prophage e14 region	over WT	others
ydhJ_4	PCO_85	3,85	0,69	0,02485	putative protein YdhJ	over WT	others
hibch_2	PCO_153	7,06	0,70	0,01021	3-hydroxyisobutyryl-CoA hydrolase%2C mitochondrial	over WT	aminoacid metabolism
mmgC_4	PCO_153	6,93	0,70	0,00907	Acyl-CoA dehydrogenase	over WT	fatty acid metabolism metabolism
45	PCO_88	4,29	0,70	0,03036	Protein gp45	over WT	Others
O	PCO_115	3,94	0,73	0,00224	Presumed capsid-scaffolding protein	over WT	Others
46	PCO_88	3,95	0,75	0,03893	Protein gp46	over WT	others
ymfQ	PCO_88	4,88	0,78	0,00479	putative protein YmfQ in lambdoid prophage e14 region	over WT	others
pfeA	PCO_103	10,86	0,80	0,04442	Ferric enterobactin receptor precursor	over WT	membrane protein
nahR_4	PCO_124	4,96	1,01	0,00081	HTH-type transcriptional activator NahR	over WT	regulation of transcrip
gabP_2	PCO_97	5,51	1,40	0,00000	GABA permease	over WT	transporter activity

Appendix VII Grouping and relative percentage of differentially regulated genes (P ≤0.05) and of the GL2 and GLRFIA mutant derivatives in comparison to wild type strains of *P. corrugata* CFBP5454 into role categories

	Transcript level in GL2 mutant versus wild type				**Transcript level in GLRFIA mutant versus wild type**			
	Up regulated		Down regulated		Up regulated		Down regulated	
Role Categories	n	%	n	%	n	%	n	%
quorum sensing	0	0.00	1	0.66	0	0.00	0	0.00
fatty acid metabolism	1	0.66	2	1.32	1	0.77	3	2.31
membrane protein	1	0.66	2	1.32	3	2.31	3	2.31
carbohydrate metabolic process	0	0.00	4	2.63	0	0.00	2	1.54
Purine and pirimidine metabolism	0	0.00	4	2.63	1	0.77	4	3.08
aminoacid metabolism	3	1.97	2	1.32	1	0.77	1	0.77
stress response	0	0.00	7	4.61	0	0.00	1	0.77
Others	3	1.97	6	3.95	12	9.23	11	8.46
regulation of transcription	3	1.97	8	5.26	5	3.85	3	2.31
alginic acid biosynthetic process	0	0.00	12	7.89	0	0.00	12	9.23
Secondary metabolite production	1	0.66	11	7.24	1	0.77	11	8.46
Redox and Oxidative stress	6	3.95	17	11.18	8	6.15	11	8.46
unknown	16	10.53	8	5.26	5	3.85	4	3.08
transporter activity	13	8.55	21	13.82	14	10.77	13	10.00
	47		105		51		79	

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