Molecular Plant Pathology

MOLECULAR PLANT PATHOLOGY (2015) **16**(5), 495–506 DOI: 10.1111/mpp.12207

Pseudomonas corrugata crpCDE is part of the cyclic lipopeptide corpeptin biosynthetic gene cluster and is involved in bacterial virulence in tomato and in hypersensitive response in *Nicotiana benthamiana*

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SUMMARY

Pseudomonas corrugata CFBP 5454 produces two kinds of cyclic lipopeptides (CLPs), cormycin A and corpeptins, both of which possess surfactant, antimicrobial and phytotoxic activities. In this study, we identified genes coding for a putative non-ribosomal peptide synthetase and an ABC-type transport system involved in corpeptin production. These genes belong to the same transcriptional unit, designated *crpCDE*. The genetic organization of this locus is highly similar to other *Pseudomonas* CLP biosynthetic clusters. Matrix-assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS) analysis revealed that transporter and synthetase genomic knock-out mutants were unable to produce corpeptins, but continued to produce cormycin A. This suggests that CrpCDE is the only system involved in corpeptin production in *P. corrugata* CFBP 5454. In addition, phylogenetic analysis revealed that the CrpE ABC transporter clustered with the transporters of CLPs with a long peptide chain. Strains depleted in corpeptin production were significantly less virulent than the wild-type strain when inoculated in tomato plants and induced only chlorosis when infiltrated into *Nicotiana benthamiana* leaves. Thus, corpeptins are important effectors of *P. corrugata* interaction with plants. Expression analysis revealed that *crpC* transcription occurs at high cell density. Two LuxR transcriptional regulators, PcoR and RfiA, have a pivotal role in *crpC* expression and thus in corpeptin production.

Keywords: ABC transporters, corpeptins, Lux R transcriptional regulators, non-ribosomal peptide synthetase, *Pseudomonas*.

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INTRODUCTION

Pseudomonas corrugata Roberts and Scarlett 1981 emend. Sutra *et al*., 1997 is a ubiquitous bacterium isolated from a wide variety of sources. It was first described in the UK in the late 1970s (Scarlett *et al*., 1978) as the causal agent of tomato pith necrosis (TPN), and later associated worldwide with TPN in tomato (Catara, 2007). The most characteristic symptom of the disease is the discoloration and/or necrosis of the parenchymatous tissue of the stem. The injection of a high-density inoculum within the stem of a wide range of plant species leads to pith necrosis (Catara *et al*., 1997, 2002; Siverio *et al*., 1993; Sutra *et al*., 1997). However, the disease is only widespread in tomato, with just a few cases of infection reported in pepper and chrysanthemum (Catara, 2007).

The hypersensitive response (HR) in tobacco has been reported as a variable test in *P. corrugata* identification/characterization (Catara *et al*., 1997; Siverio *et al*., 1993; Sutra *et al*., 1997). A number of studies by Gustine *et al*. (1990, 1994, 1995) have shown that *P. corrugata* strain 388 causes HR in tobacco leaves and elicits phytoalexin (medicarpin) biosynthesis in white clover callus, K^+/H^+ exchange in tobacco leaf discs and an active oxygen burst in white clover suspension cultures. However, surprisingly, in the genome of the *P. corrugata* strain CFBP 5454, no Hrp1 type III secretion system (T3SS) has been found (Licciardello *et al*., 2014).

Pseudomonas corrugata produces cyclic lipopeptide (CLP) corpeptins; two isoforms consisting of 22-amino-acid residues, corpeptin A and corpeptin B, have been described. 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 cormycin, a lipodepsinonapeptide, which has antimicrobial activity against *B. megaterium* and also against the yeast *Rhodotorula pilimanae*. Cormycin also exhibits phytotoxic activity, inducing chlorosis followed by necrosis in tobacco (Scaloni *et al*., 2004). CLPs produced by *Pseudomonas* spp. are composed of a

fatty acid tail linked to a short oligopeptide, which is cyclized to form a lactone ring between two amino acids in the peptide chain (Raaijmakers *et al*., 2006). The corpeptin structure, as determined by Emanuele *et al*. (1998), is strictly related to peptin-like CLPs, which are characterized by long peptide chains ranging from 18 to 25 amino acids. These CLPs include fuscopeptins, produced by the phytopathogen *P. fuscovaginae* (Ballio *et al*., 1996), syringopeptins, synthesized by phytopathogenic strains of *P. syringae* pv. *syringae* (Ballio *et al*., 1991), and tolaasin, produced by the mushroom-infecting bacterium *P. tolaasii* (Coraiola *et al*., 2006). Cormycin belongs to the group of smaller nonapeptides (Scaloni *et al*., 2004), with syringomycin, produced by strains of *P. syringae* pv. *syringae*, having been the most extensively studied (Bender *et al*., 1999; Raaijmakers *et al*., 2006).

Like many other biologically active secondary metabolites, CLPs are synthesized by multifunctional non-ribosomal peptide synthetases (NRPSs) (Finking and Marahiel, 2004); Raaijmakers *et al*., 2006). NRPSs have a modular structure, and each module is a building block resulting in the stepwise incorporation of one amino acid in the peptide chain (Finking and Marahiel, 2004). Each amino acid activation module has a minimal set of three domains: an aminoacyl-adenylation (A) domain, a thiolation (T) domain and a condensation (C) domain (Finking and Marahiel, 2004). The genetic organization of CLP biosynthetic loci reveals that several genes flanking the biosynthetic genes are conserved among *Pseudomonas* spp. Genes coding for putative CLP transporters, located downstream of the last synthetase gene, and regulatory genes positioned up- and downstream of the CLP biosynthesis genes, have been identified and described in a number of clusters (de Bruijn and Raaijmakers, 2009; Gross and Loper, 2009).

Our previous studies have demonstrated that *P. corrugata* CFBP 5454 possesses an *N*-acyl-homoserine lactone quorum sensing (AHL-QS) system, PcoI/PcoR, consisting of an AHL synthase, PcoI, and a transcriptional sensor/regulator belonging to the LuxR family protein, PcoR (Licciardello *et al*., 2007). Downstream of *pcoI* is *rfiA*, which is co-transcribed with *pcoI* (Licciardello *et al*., 2009). RfiA belongs to LuxR regulators whose particular characteristic is that they contain the typical helix–turn–helix (HTH) motif at the C-terminus, but do not harbour the autoinducer-binding terminus typical of QS LuxR regulators (de Bruijn and Raaijmakers, 2009). PcoR activates *pcoI* expression in the presence of AHL via a typical positive-feedback regulatory loop. As *pcoI* and *rfiA* constitute an operon, the expression of *rfiA* is directly regulated by the PcoR–AHL complex (Licciardello *et al*., 2009). *Pseudomonas corrugata pcoR* and *rfiA* mutants show significantly reduced virulence when inoculated in tomatoes. CLPs were also absent in their culture filtrates (Licciardello *et al*., 2009, 2012). Genetically linked to *rfiA* is the operon *pcoABC*, which is under positive regulation by RfiA, and thus indirectly by the PcoI/R system (Licciardello *et al*., 2009). A null mutant in *pcoABC* retains the ability to inhibit the growth of CLP indicator microorganisms *R. pilimanae* and *B. megaterium*, although at a reduced level compared with the wild-type (WT), and is as virulent as the WT strain. These findings indicate that other CLP secretion mechanisms must exist in *P. corrugata*. The aforementioned genes (*pcoI/pcoR*, *rfiA* and *pcoABC*) are located in a cosmid insert of approximately 20 000 bp (Licciardello *et al*., 2009). The insert sequence also supports the presence of genes putatively coding for an additional ABC transporter and part of an NRPS highly homologous to genes of other *Pseudomonas* spp. CLP biosynthetic clusters.

In this article, we demonstrate that these ABC transporter genes (*crpDE*) form an operon with the last gene of a putative NRPS (*crpC*), and that both are part of the biosynthesis cluster of *P. corrugata* corpeptins, which are demonstrated to have an important role in plant interaction and in virulence. A role for the transcriptional regulators PcoR and RfiA in corpeptin production and *crpC* gene expression is also demonstrated.

RESULTS

Analysis of a putative ABC efflux system and a putative NRPS in *P. corrugata*

Recently, we have demonstrated that the AHL-QS system of *P. corrugate*, PcoI/PcoR, and the transcriptional regulator RfiA play an important role in the regulation of virulence via CLP production (Licciardello *et al*., 2012). The absence of further genetic and molecular information prompted us to sequence the complete cosmid insert from which the AHL-QS genes were identified (Fig. 1). Three complete and one partial new open reading frames (ORFs) were identified. ORF8 spans 1152 bases and codes for a

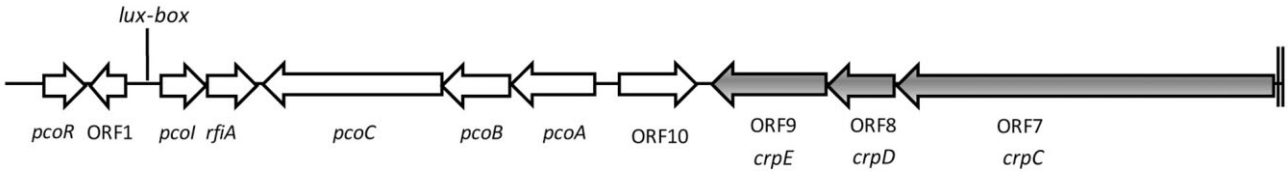

Fig. 1 Representation of an approximately 21-kb DNA region of *Pseudomonas corrugata* CFBP 5454 harbouring the *N*-acyl-homoserine lactone quorum sensing (AHL-QS) system genes (*pcoI/pcoR*), the transcriptional regulator *rfiA* belonging to the LuxR-type transcriptional regulators, the *pcoABC* operon, coding for a resistance nodulation–cell division transporter system, the *crpDE* operon (i.e. ORF8, 9) coding for an ATP-binding transporter system and *crpC* (ORF7) which is part of a non-ribosomal peptide synthetase.

putative 383-amino-acid protein which displays a high level of amino acid sequence similarity (81%–91%) to periplasmic membrane fusion proteins of other *Pseudomonas* species. Downstream of this ORF, separated by only 2 bp, and transcribed in the same direction, ORF9 spans 1962 bases and encodes a predicted 653-amino-acid protein. BLASTX analysis of *P. corrugata* ORF9 revealed homologies with a number of cytoplasmic ABC-type transporters of *Pseudomonas* species, with a similarity ranging from 90% to 85%. Downstream of ORF9 was ORF10, which is separated by 299 bp and is transcribed in the opposite direction. ORF10 codes for a putative diaminobutyrate-2-oxoglutarate transaminase protein (ORF10), which displays 80% identity to *P. syringae* B728a (YP_235696.1). Located 78 bp upstream of ORF8 and translated in the same direction, we identified a truncated ORF (ORF7). The 6471 bases encode 2156 amino acids which show high homology (66%–50%) to members of NRPS genes involved in the CLP biosynthesis of other *Pseudomonas* spp., e.g. arthrofactin synthetase C (BAC67536.1), massetolide MassC (ABH06369.2) and orfamide NRPS OfaC (YP_259254.1). Analysis of the deduced amino acid sequence for the C-terminal portion of the ORF7 protein revealed the presence of an entire amino acid activation module, containing conserved core sequences for the C, A and T domains, and a truncated amino acid activation module lacking the C domain. Two thioesterase TE domains, each containing the conserved GxSxG sequence motif involved in the linear or cyclic peptide release, were also identified (Gross and Loper, 2009). Tandem TE domains have been described in the termination modules of biosynthetic clusters of syringopeptin, arthrofactin, viscosin, massetolide, orfamide, putisolvin and entolysin (de Bruijn *et al*., 2008; Dubern *et al*., 2008; Gross *et al*., 2007; Roongsawang *et al*., 2003; Scholz-Schroeder *et al*., 2003; Vallet-Gely *et al*., 2010).

The presence of genes flanking the terminating NRPS genes, encoding components of exporter systems, is a recurrent theme for several *Pseudomonas* CLP clusters. We therefore analysed the genomic context of each ORF9 homologue (ABC transporter-encoding) within plant-associated *Pseudomonas* spp. Genes coding for all of these ABC transporters were localized downstream of the last putative NRPS genes as deduced by bioinformatics (Table S1, see Supporting Information). The neighbour-joining dendrogram of these ABC transporters aligned by CLUSTALW in MEGA 5.01 software resulted in distinct clusters (Fig. 2). *Pseudomonas corrugata* ORF9 formed a cluster together with ABC transporters of *P. syringae* pv. *syringae* strains 642 (ZP_07265130.1) and B728a (YP_235695.1), located downstream of genes coding for syringopeptins (Scholz-Schroeder *et al*., 2003), and those of *P. fuscovaginae* UPB 0736 (ZP_10991311.1), *P. syringae* pv. *avellanae* ISPaVe013 (ZP_17805054) and *Pseudomonas* sp. CMR12a (AFH75324.1). Another well-delineated cluster group of transporters located downstream of NRPSs includes SyfD, involved in the export of the syringafactin linear lipopeptide of *P. syringae* pv. *tomato* DC3000 (NP_792636.1) (Berti *et al*., 2007), and other ABC transporters in *P. syringae* pathovars (Baltrus *et al*., 2011), annotated as syringolide efflux proteins (EGH63888.1; EGH94284.1; EGH09011.1), but which, in our analysis, were located downstream of NRPS genes highly homologous to syringafactins and annotated as *sifB* (Table S1). The remaining ABC transporters belong to the biosynthesis gene

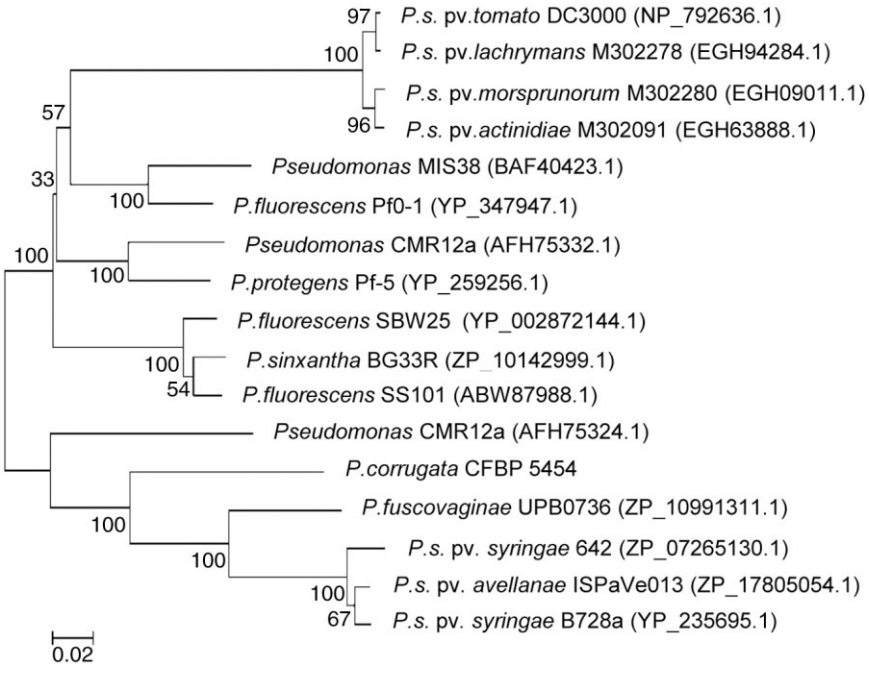

Fig. 2 Phylogenetic analysis of amino acid sequences of 17 ABC-type transporters identified in cyclic lipopeptide (CLP) biosynthetic clusters of plant-associated *Pseudomonas* spp. The numbers at the nodes indicate the level of bootstrap support, based on neighbour-joining analysis of 500 re-sampled datasets. The bar indicates the relative number of substitutions per site. The protein Accession numbers are given in parentheses.

clusters of the medium-chain-length peptide CLPs arthrofactin (BAF40423.1), massetolide (ABW87988.1), motilin (AFH75332.1), viscosin (YP_002872144.1) and orfamide (YP_259256.1).

crpCDE is part of the corpeptin biosynthetic cluster

In order to examine the role of these newly identified ORFs in CLP biosynthesis, *P. corrugata* ORF7 and ORF8 were insertionally inactivated. The two genomic mutant strains were designated as PCONRPS and PCOMFP, respectively (Table 1). Matrix-assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS) analysis of CFBP 5454 cell-free culture filtrates used as controls highlighted the presence of cormycin and corpeptins, corresponding to specific peaks at *m/z* 1274, 2095.3 and 2121.2, respectively (Licciardello *et al*., 2012). Analysis of the cell-free culture filtrates of the aforementioned knock-out mutants grown in CLP-inducing conditions revealed that the two mutants no longer produced corpeptins, whereas they continued to produce cormycin, suggesting that the knocked-out genes are involved in corpeptin production (Fig. 3A). This also suggested that CrpDE was probably involved in the secretion of corpeptins and that CrpC was part of the NRPS complex for the biosynthesis of this CLP. The bioactivity of the culture filtrates of the two mutants was tested using an assay based on the inhibition of the *in vitro* growth of the Gram-positive bacterium *B. megaterium* and the yeast *R. pilimanae*. The inhibitory activity of PCOMFP and PCONRPS strain cell-free culture filtrates was significantly reduced against both microorganisms compared with that of the parent strain (Fig. 3B).

Based on genotypic and phenotypic evidence, we designated ORF7, ORF8 and ORF9 as *crpC*, *crpD* and *crpE*, respectively, and PCONRPS and PCOMFP as *crpC*::pKnock and *crpD*::pKnock,

Table 1 Bacterial strains, plasmids and oligonucleotides used in this study.

Strain	Genotype/relevant characteristics	Reference or source
Pseudomonas corrugata		
CFBP 5454	Wild-type, source of *crpC* and *crpD*	CFBP
PCOMFP	*crpD*:: pKnock, Km^r	This study
PCONRPS	*crpC*:: pknock, Km^r	This study
PCOMFPC	PCOMFP complemented with pBBR-CrpDE	This study
GL2	*pcoR*76::Tn5, Km^r	Licciardello *et al*. (2007)
GLRFIA	*rfiA*:: pKnock, Km^r	Licciardello *et al*. (2009)
Escherichia coli		
pLC3.34	pLAFR3 containing *P. corrugata* CFBP 5454 DNA, Tc^r	DISPA
DH5α	F2 f80d*laczZDM15* D(*lacZYA-argF*)*U169 endA1 recA1 hsdR17 deoR gyrA96 thi-1 relA1 supE44*	Sambrook *et al*. (1989)
CC118λ*pir*	Δ(*ara, leu*)*7697 araD139* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB* (Rf^r) *argE*(Am) *recA1* λ*pir*	Herrero *et al*. (1990)
Chromobacterium violaceum		
CV026	ATCC 31532 derivative, *cvil*::Tn5*xylE* Km^r Sm^r	McClean *et al*. (1997)
Plasmids		
pCR2.1	Cloning vector TA, Amp^r	Invitrogen
pGEM-T	Cloning vector TA, Amp^r	Promega
pKNOCK-Km^r	Mobilizable suicide vector, Km^r	Alexeyev (1999)
pRK2013	Km^r100, Tra^+ Mob^+ ColE1 replicon	Figurski and Helinski (1979)
pKMMfp	pKNOCK containing an internal fragment of *P. corrugata* CFBP 5454 *corpD* gene	This study
pKMNrps	pKNOCK containing an internal fragment of *P. corrugata* CFBP 5454 *corpC* gene	This study
pBBR-CrpDE	pBBR1MCS-5 containing the full-length *P. corrugata* CFBP 5454 *crpDE* genes	This study
Oligonucleotides	**Sequence**	
MFPkn-fw	5'-AAGGATCCAGTGGCTGGCGGAAATC-3'	This study
MFPkn-rev	5'-GGTCTAGAGGATGGTGAAATACACT-3'	This study
NRPSkn-fw	5'-CAGGATCCGGATCTATCTGCTCGAC-3'	This study
NRPSkn-rev	5'-AATCTAGAGCCGATAGTGCCGAGGG-3'	This study
PCR1-fw	5'-ACCGCAACATCAATACAGCG-3'	This study
PCR1-rev	5'-ACCGACATCAACCTGCTTGAC-3'	This study
PCR2-fw	5'-CATCGCCTGCGTATCTCGAT-3'	This study
PCR2-rev	5'-CAACTCATGGTCGTCCATCG-3'	This study
Pco16S fw	5'-TGTAGCGGTGAAATGCGTAGAT-3'	Conte *et al*. (2006)
Pco16S rev	5'-CCTCAGTGTCAGTATCAGTCCAG-3'	Conte *et al*. (2006)
Abc1-fw	5'-CAAAATCGCTATCGTGCTTGTC-3'	This study
Abc1-rev	5'-CGACCGTAGCGGTCAGGTA-3'	This study
Nrps-fw	5'-ACGGGCCACCCGAAAG-3'	This study
Nrps rev	5'-GAGGCGAAAGCCACGTGAT-3'	This study
ABC-fw	5'-AAGCTTTGGGCACACCCATG-3'	This study
ABC-rev	5'-GGATCCCACAGAGGACAGTG-3'	This study
Primerseq-fw	5'-GATCCATCGACGGACTGTC-3'	This study
Primerseq-rev	5'-CATCGTGTTCGGTTTCGTAC-3'	This study

CFBP, Collection Francaise de Bacteries Phytopathogenes, Angers, France; DISPA, Dipartimento di Scienze delle Produzioni Agrarie ed Alimentari, University of Catania, Catania, Italy.

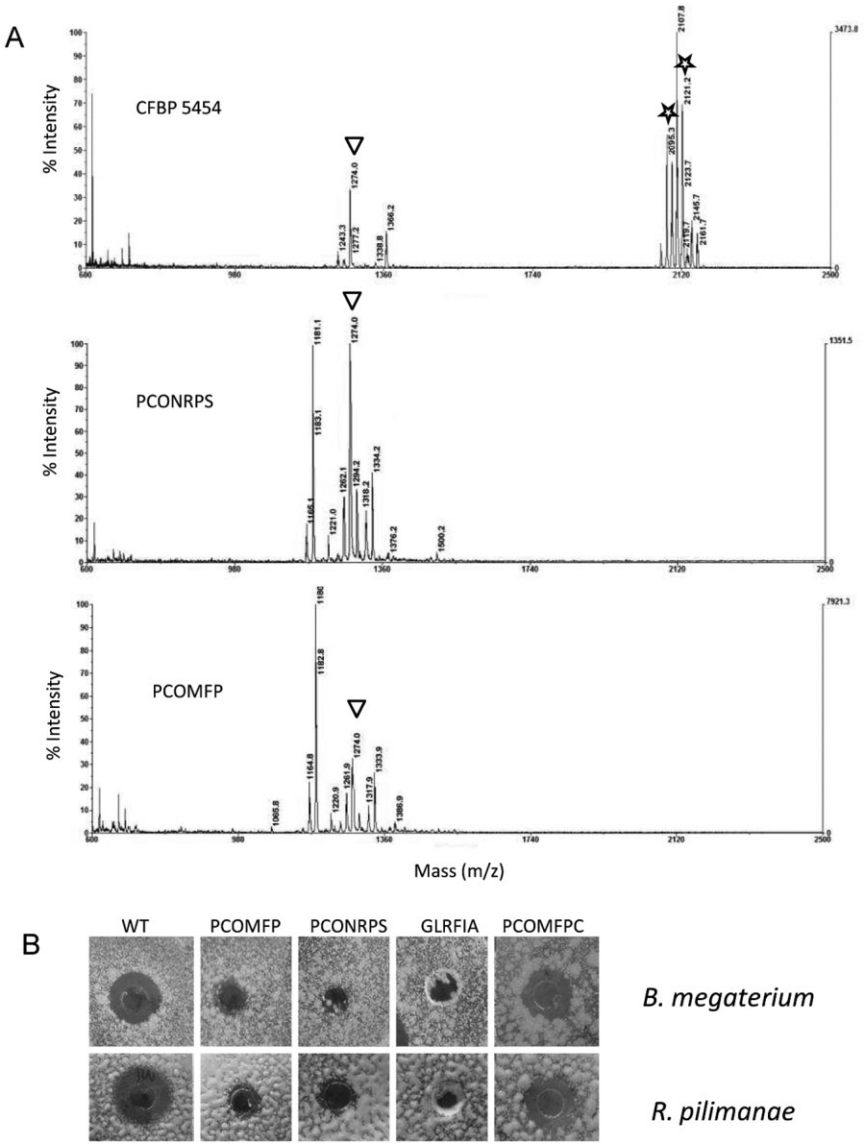

Fig. 3 Analysis of cell-free extracts of *Pseudomonas corrugata* strains after 96 h of incubation at 28 °C: CFBP 5454 (wild-type, WT); PCONRPS (*crpC*::pKnock); PCOMFP (*crpD*::pKnock). (A) Representative matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectra. The triangles indicate the peaks corresponding to cormycin and the stars indicate those corresponding to corpeptins. (B) Antimicrobial assay to evaluate the bioactivity of cell-free culture filtrates; the GLRFIA mutant was included as a negative control; PCOMFPC, PCOMFP strain complemented *in trans* with intact *crpDE*.

respectively. In addition, the three genes constitute an operon, designated *crpCDE*, as determined by a reverse transcription approach with two sets of primers: PCR1 to capture the intervening region between *crpC* and *crpD*, upstream of the knock-out inactivation site of PCOMFP, and PCR2 which amplifies the intervening region between *crpD* and *crpE* (Fig. 4). This result was also confirmed by quantitative real-time polymerase chain reaction (Q-PCR) assays, which showed that *crpC* transcription occurred in the PCOMFP strain, whereas *crpD* expression was undetectable in the PCONRPS strain (data not shown).

Corpeptins contribute to *P. corrugata*–plant interaction

To investigate the role of *crpCDE* in *P. corrugata*–plant interaction, we set up *in planta* assays. The tests were performed with the PCOMFP and PCONRPS mutant strains (producing only cormycin) and, as controls, the parent strain CFBP 5454 (which produces both cormycin and corpeptins) and the GLRFIA mutant strain (*rfiA*$^{-}$) (which is no longer able to produce both cormycin and corpeptins) (Licciardello *et al*., 2012).

Fifteen days after inoculation, stem pith-infected tissues of tomato plants inoculated with the parent strain showed dark brown lesions ranging in length from 3 to 6 cm, necrotic in the inner part and at times hollow (Figs 5 and 6A). Again, the GLRFIA mutant strain did not cause any lesions on tomato stem pith other than a pale discoloration at the inoculation site. The plants inoculated with PCOMFP and PCONRPS mutant strains showed a reduced brown discoloration of the stem pith (approximately 1–2 cm) (Figs 5 and 6A). At 3 and 7 days post-inoculation (dpi), CFBP 5454 showed a bacterial titre of approximately 10^{12} colony-forming units (cfu)/cm of stem at the inoculation site, whereas the two derivative strains showed a titre of approximately 10^{9} cfu/cm

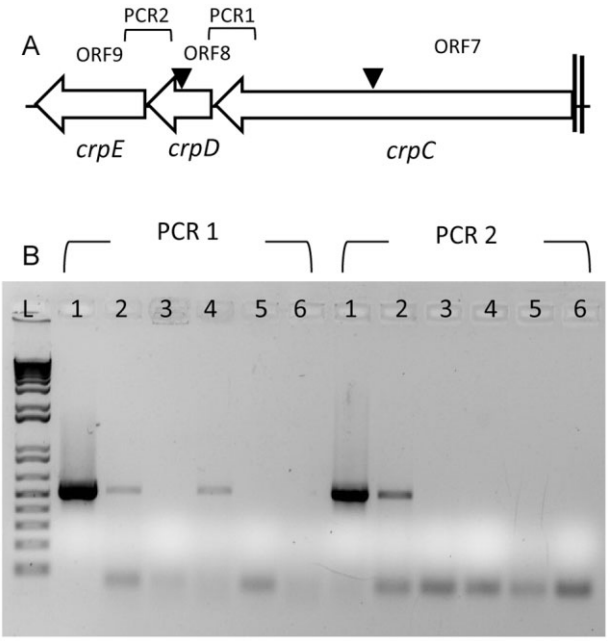

Fig. 4 Polycistronic organization of *crpCDE* cluster identified in *Pseudomonas corrugata* CFBP 5454. (A) Triangles indicate the positions of insertional mutagenesis. The brackets indicate the positions of primer sets overlapping *crpC* and *crpD* (PCR1) and *crpD* and *crpE* (PCR2). (B) Agarose gel electrophoresis of reverse transcription-polymerase chain reaction (RT-PCR) analysis performed with total RNA isolated from *P. corrugata* CFBP 5454 and mutant strains incubated on Improved Minimal Medium (IMM) for 4 days at 28 °C. Two sets of primers (PCR1 and PCR2) were designed for the amplification of approximately 500-bp DNA fragments at each inter-gene locus gap. Lanes: L, 1-kb plus ladder (Life Technologies, Carlsbad, CA, USA); 1, wild-type (WT) genomic DNA; 2, WT cDNA; 3, *crpC*::pKnock; 4, *crpD*::pKnock; 5, negative control of the reverse transcription step; 6, PCR negative control, water.

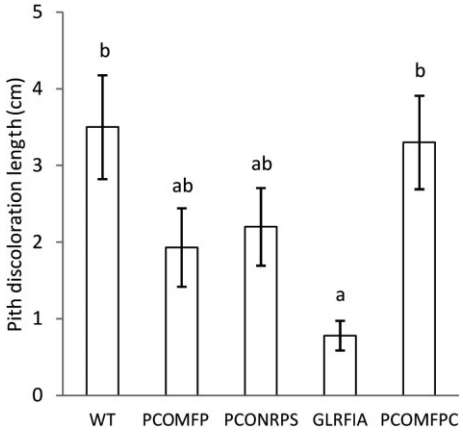

Fig. 5 Length of pith discoloration in tomato plants inoculated with *Pseudomonas corrugata* CFBP 5454 (wild-type, WT), PCOMFP (*crpD*::pKnock), PCONRPS (*crpC*::pKnock), GLRFIA (*rfiA* mutant) and PCOMFPC (PCOMFP strain complemented *in trans* with intact *crpDE*). Values are means ± standard error (SE) of 20 replicates. Values followed by the same letters do not differ significantly at $P \leq 0.05$ according to Student–Newman–Keuls test. The results presented are representative of two independent experiments.

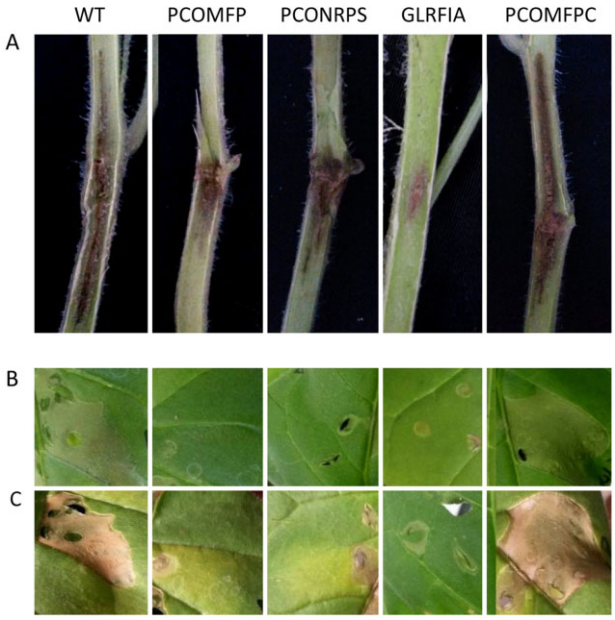

Fig. 6 *In planta* inoculations of *Pseudomonas corrugata* CFBP 5454, PCOMFP (*crpD* mutant), PCONRPS (*crpC* mutant), GLRFIA (*rfiA* mutant) and PCOMFPC (PCOMFP strain complemented *in trans*). (A) Stem pith necrosis symptoms in prick-inoculated tomato plants. (B, C) Hypersensitivity reaction on tobacco leaves at 24 h (B) and 96 h (C) post-inoculation with bacterial suspension. These photographs are representative of at least two experiments with replicates.

of stem (Table 2). Monitoring at different distances above and below the inoculation site always revealed the presence of all three strains. Three-way analysis of variance (ANOVA) showed significant effects of strains ($P < 0.001$) and distances ($P < 0.001$), but not of positions or interaction effects. The population concentration of the three strains varied significantly at each sampling time and with the following order CFBP 5454 > PCONRPS > GLRFIA (Table 2). The bacterial titre declined significantly with increasing distance from the inoculation site both below and above the inoculation site (Table 2).

We have observed previously that both GL2 (*pcoR*$^{-}$) (Licciardello *et al*., 2007) and GLRFIA (V. Catara, University of Catania, Catania, unpublished data) mutant strains do not cause any necrosis symptoms when inoculated in *Nicotiana tabacum* and *N. benthamiana* leaf mesophyll, in contrast with the WT CFBP 5454 strain, which causes the collapse of the infiltrated panels within 24 h, followed by necrosis. To investigate the possible influence of corpeptin production in such leaf necrosis, we infiltrated *N. benthamiana* leaf panels with a bacterial suspension of the parent strain CFBP 5454 and derivative mutants. Twenty-four hours after inoculation, the leaf panels inoculated with the parent strain showed a collapsed mesophyll, which turned necrotic within the following 24 h (Fig. 6B). At the same scoring times, leaf panels inoculated with

Table 2 *Pseudomonas corrugata* strain CFBP 5454 and derivative mutant strain PCONRPS and GLRFIA population concentrations (log cfu/cm) in tomato stem 7 days after inoculation at different distances above and below the inoculation sites*.

Strain	Position	Strain population concentration at different distances from the inoculation site (0)†				Average position/strains†	Average strains†
		0	1 cm	2 cm	3 cm		
CFBP5454	Above	12.44 ± 0.01	7.73 ± 0.57	6.50 ± 0.40	5.27 ± 0.37	6.50 c	
	Below		7.29 ± 0.17	7.64 ± 0.38	6.49 ± 0.34	7.14 c	
	Mean		7.51 c	7.07 c	5.88 c		6.81 c
PCONRPS	Above	9.86 ± 0.20	6.34 ± 0.36	5.90 ± 0.14	4.81 ± 0.14	5.68 b	
	Below		6.72 ± 0.13	5.61 ± 0.10	4.40 ± 0.07	5.58 b	
	Mean		6.53 b	5.75 b	4.60 b		5.63 b
GLRFIA	Above	9.42 ± 0.19	5.30 ± 0.30	4.11 ± 0.37	3.20 ± 0.30	4.20 a	
	Below		5.36 ± 0.09	4.56 ± 0.11	3.39 ± 0.33	4.44 a	
	Mean		5.33 a	4.33 a	3.29 a		4.32 a
Average distances‡			6.46 c	5.72 b	4.59 a		
Average positions†		Above	5.46 a				
		Below	5.72 a				

*A three-way analysis of variance (ANOVA) was performed to investigate differences in bacterial population concentration of the three strains and expressed as log cfu/cm of stem sampled at different distances (1, 2 and 3 cm) from and positions (above and below) the inoculation sites (values are means of six replicates ± standard error). Three-way ANOVA showed significant effects of strains ($n = 36$; $P < 0.001$), distances ($n = 36$; $P < 0.001$) but not positions ($n = 54$; $P = 0.072$).
†Averages followed by the same letter within columns are not significantly different according to Student–Newman–Keuls test ($P = 0.01$).
‡Averages of strain bacterial populations at different distances are compared within the row. This experiment was repeated on two separate dates with similar results.

PCOMFP and PCONRPS showed a healthy turgid appearance (Fig. 6B), comparable with the water-inoculated control (data not shown). Four days after inoculation, chlorosis was evident in the leaf panels inoculated with PCOMFP and PCONRPS, but not with GLRFIA (Fig. 6C). In a time course experiment, we monitored the bacterial population titres of strain CFBP 5454 and the PCONRPS derivative mutant in infiltrated *N. benthamiana* leaf panels (Fig. 7). We included the GLRFIA mutant and a *P. syringae* pv. *tabaci* strain (DAPP-PG632) in the trial as pathogenic controls. In tissues infiltrated with *P. syringae* pv. *tabaci*, the bacterial population increased drastically to 10^{10} cfu/leaf disc, whereas the *P. corrugata* strain CFBP 5454 population began to decay from the first dpi to 10^4 cfu/leaf disc. The bacterial population in leaf panels inoculated with PCONRPS and GLRFIA mutants maintained the same bacterial population titres (e.g. 10^6 cfu/leaf disc) from the first sampling at 0 dpi throughout the trial. At all sampling dates from 1 dpi these two strain titres were significantly different from those of strain CFBP 5454 ($P < 0.01$). Expression *in trans* of *crpDE* in the PCOMFP mutant by the introduction of pBBR-CrpDE restored both virulence and antimicrobial activity similar to the WT strain level, as well as the HR-positive phenotype (Figs 5 and 6).

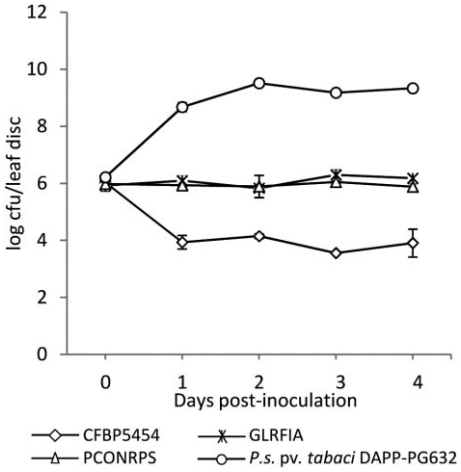

Fig. 7 *Pseudomonas corrugata* strain CFBP 5454 and derivative mutant strain PCONRPS and GLRFIA population concentrations in *Nicotiana benthamiana* leaves Three *N. benthamiana* leaf discs were collected independently and processed at each time point (0, 1, 2, 3 and 4 days post-inoculation). In this trial, *Pseudomonas syringae* pv. *tabaci* strain DAPP-PG632 was used as the pathogenic control strain. In both trials, samples were homogenized with 1 mL of distilled water; serial dilutions were plated onto NDA medium (nutrient agar plus 1% dextrose) plus antibiotics as described in Experimental procedures. Values are means ± standard error (SE) of multiple replicates. The results presented are representative of two independent experiments.

LuxR transcriptional regulators PcoR and RfiA are involved in *crpCDE* expression

Pseudomonas corrugata mutants in the genes coding for the transcriptional regulators *pcoR* (GL2 mutant) and *rfiA* (GLRFIA mutant) no longer produced CLPs (Licciardello *et al*., 2012). By investigating the expression of *crpC* and *crpDE* in both the GL2 and GLRFIA mutant strains, we observed that transcript levels were significantly and consistently reduced in both mutant strains (data not shown). These results suggest that, under the conditions tested, both transcriptional regulators play a role in corpeptin biosynthesis/secretion in *P. corrugata*.

CLP production, evaluated on the basis of antimicrobial activity against the two CLP-sensitive bioindicators *R. pilimanae* and *B. megaterium*, occurs in a cell density-dependent manner with a trend similar to AHL production (Licciardello *et al*., 2009).

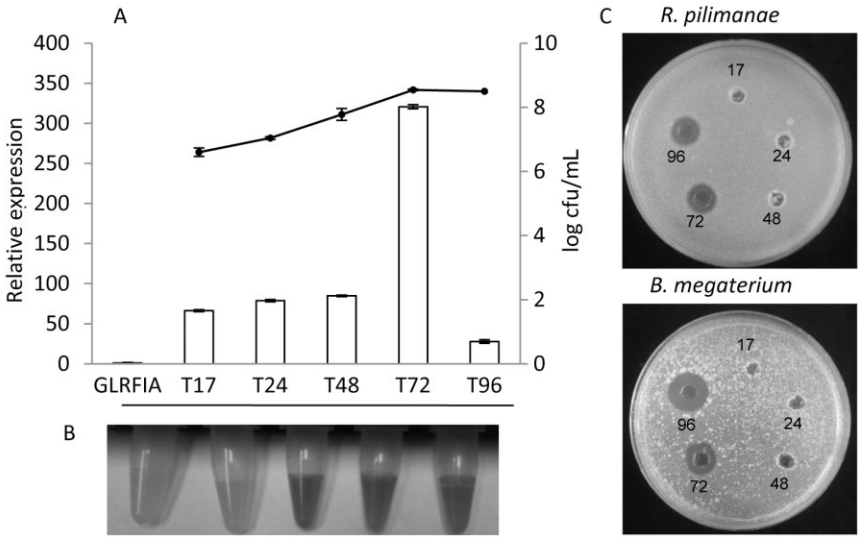

Fig. 8 Cyclic lipopeptide (CLP) production in *Pseudomonas corrugata* CFBP 5454 during bacterial growth. (A) Time-course expression (17, 24, 48, 72 and 96 h post-inoculation) of *crpC* in *P. corrugata* CFBP 5454 grown in CLP production-inducing conditions (bar graph). Results are reported as the fold difference relative to data obtained for the non-producing corpeptin mutant strain GLRFIA. Values are means calculated from duplicate samples from three RNA extractions ± standard error (SE). The CFBP 5454 growth curve is displayed in the line graph (secondary *y* axis). (B, C) The cell-free culture filtrates at each time point were tested by bioassays for *N*-acyl-homoserine lactone (AHL) production using *Chromobacterium violaceum* CV026 as biosensor to detect AHLs (B) and antimicrobial activity against the yeast *Rhodotorula pilimanae* and the Gram-positive bacterium *Bacillus megaterium* (C).

Consequently, we speculated whether *crpC* transcriptional levels would follow this trend in a time-course experiment. Bacterial cell samples were recovered from still cultures grown in CLP-inducing medium at 17, 24, 48, 72 and 96 h post-inoculation (hpi) (i.e. T17, T24, T48, T72 and T96). During growth of the WT strain, transcript levels of *crpC* were similar between 17 and 48 h, whereas they reached a maximum after 72 h of growth and decreased consistently 96 h after inoculation (Fig. 8A). In cell-free culture filtrates sampled at the same time points, MALDI-TOF analysis detected small amounts of corpeptins (but also cormycin) starting from T48, when the cell concentration was approximately as low as 7×10^8 cfu/mL (data not shown). At the same sampling time (T48), the presence of AHL was detected for the first time by indirect violacein production by the biosensor *Chromobacterium violaceum* CV026 cultures (Fig. 8B). The sampling of 72-h-old cultures highlighted high CLP peak signals in MALDI-TOF mass spectra and the first evidence of *in vitro* antimicrobial activity (Fig. 8C).

DISCUSSION

In this study, we identified part of the biosynthetic cluster responsible for corpeptin production, including genes transcriptionally joined coding for an NRPS and an ABC efflux system, designated *crpCDE*. We demonstrated that these genes, and hence corpeptins, greatly contribute 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 (Gross and Loper, 2009). It is estimated that approximately 3 kb of DNA are required to code for each amino acid activation module (Gross and Loper, 2009); thus, it is possible to predict that the corpeptin NRPS system encompasses approximately 75 kb of DNA to code the 22-amino-acid activation modules in the same way as with the *P. syringae* pv. *syringae* syringopeptin biosynthetic cluster, which is the largest linear NRPS system described for prokaryotes (Scholz-Schroeder *et al*., 2003).

Recently, we have reported the whole-genome shotgun sequencing of *P. corrugata* CFBP 5454, and an initial analysis revealed that approximately 217 kb coded for putative NRPS biosynthetic clusters (Licciardello *et al*., 2014). Genes that code for these large multimodular enzymes with repetitive domain structures in next-generation sequencing approaches are difficult to assemble and typically split between several contigs. Thus, a great deal of further work is required in order to obtain the entire CLP biosynthetic clusters.

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 the biological activity of corpeptins, we 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. Similarly, in the *P. syringae* pv. *syringae* strain B01D, a *sypA* mutant that produces syringomycin (but not syringopeptins) was still able to inhibit the growth of both *B. megaterium* and *Geotrichum candidum* and was less virulent in sweet cherry fruits (Scholz-Schroeder *et al*., 2001). In *P. syringae* pv. *syringae*, three transporter systems, the SyrD (Quigley *et al*., 1993), PseABC (Kang and Gross, 2005) and PseEF (Cho and Kang, 2012) efflux systems, are mainly responsible for the secretion of the syringomycin and syringopeptins produced by strain B301D. Other natural functions of CLPs, mainly investigated in biocontrol strains, are their role in antagonism towards other (micro)organisms, motility and attachment to surfaces (Raaijmakers *et al*., 2010). Mutational analysis in CFBP 5454 mutants affected in CLP production raised interesting aspects with regard to their role in *P. corrugata* antimicrobial activity and motility that deserve further study.

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. However, we observed that 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. This result, and the fact that no T3SS was found in the *P. corrugata* CFBP 5454 genome, suggests 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).

In this work, we also showed that the transcriptional regulators PcoR and RfiA play a pivotal role in the expression of *crpC* and *crpD* genes. LuxR-type transcriptional regulators positioned up- and downstream of the CLP biosynthetic cluster also play a pivotal role in the production of syringomycin and syringopeptins (Lu *et al*., 2002; Wang *et al*., 2006), viscosin and massetolide (de Bruijn and Raaijmakers, 2009), syringafactins (Berti *et al*., 2007) and putisolvins (Dubern *et al*., 2008). *Pseudomonas corrugata* RfiA is highly homologous to a number of these CLP-associated *Pseudomonas* LuxR regulators, including *P. syringae* pv. *syringae* SalA, which is located in the *syr-syp* genomic island (Lu *et al*., 2002). SalA positively regulates all the genes of the *syr-syp* cluster associated with the biosynthesis, secretion and regulation of syringomycin and syringopeptins, and is crucial in the development of disease symptoms (Lu *et al*., 2002, 2005). The RfiA gene is regulated directly by *P. corrugata* QS by co-transcription with *pcoI*, which codes for the LuxI homologue AHL synthase (Licciardello *et al*., 2009). Similarly, in *Pseudomonas* sp. DF41, the RfiA mutant strain, in which *rfiA* is also co-transcribed with the LuxI homologue gene, was depleted of antifungal activity and showed markedly reduced CLP sclerosin production (Berry *et al*., 2014).

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. To date, the involvement of QS in CLP production has only been demonstrated in a few *Pseudomonas* strains, namely in the plant pathogen *P. fluorescens* strain 5064 and saprophytic strain *P. putida* PCL1445, where AHL QS was shown to be involved in viscosin and putisolvin biosynthesis, respectively (Cui *et al*., 2005; Dubern *et al*., 2006).

The mutagenesis of *P. corrugata* CFBP 5454 genes in this study and the bioinformatic analysis revealed that the *P. corrugata* corpeptin biosynthetic locus is highly similar to that of other CLP-producing *Pseudomonas*. Functional analysis revealed that corpeptins, long-chain CLPs, as with *P. syringae* syringopeptins, are important for virulence. As the *syr-syp* cluster in *P. syringae* represents approximately 2% of its genome, it is also important to study the biosynthesis and regulation in *P. corrugata* which, taxonomically, is more strongly related to the fluorescent oxidase-positive *Pseudomonas* biocontrol strains than to *P. syringae* pv. *syringae*. However, as, unlike *P. syringae* pv. *syringae*, QS is involved in corpeptin production via PcoR and Rfia, and *crpCDE* also seems to be involved in HR, *P. corrugata* represents an interesting and intriguing study model.

EXPERIMENTAL PROCEDURES

Bacterial strains and culture conditions

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

DNA recombinant techniques

DNA manipulations, including digestions with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments, ligation with

T4 DNA ligase, DNA hybridization, radioactive labelling by random priming and *E. coli* transformation, were performed as described by Sambrook *et al*. (1989). Southern hybridizations were performed using Amersham Hybond N+ membranes (GE Healthcare Bio-Sciences, Pittsburgh, USA). Total DNA from *Pseudomonas* spp. was isolated using the Gentra Puregene Cell Kit (Qiagen Inc., Valencia, CA, USA). Triparental matings from *E. coli* to *P. corrugata* were carried out with the helper strain *E. coli* DH5α (pRK2013) (Figurski and Helinski, 1979).

DNA sequencing and sequence analysis

Two contigs were obtained sequencing the cosmid pLC3.34 DNA insert on both strands (Macrogen, Inc., Seoul, South Korea). A set of primers, Primerseq-fw/rev, overlapping the ends of the two sequences, was used to merge the gap between the two contigs, yielding a 2284-bp amplicon (Table 1). The resulting amplicon was sequenced on both strands by BMRCRIBI (University of Padua, Italy). Homology searches of nucleotide and protein sequences were performed using the BLAST searching program in the National Center for Biotechnology Information (NCBI) database (Altschul *et al*., 1990). For phylogenetic analysis, alignments were made with CLUSTALW incorporated into the MEGA 5.01 software package (Tamura *et al*., 2011). The previous sequence, Accession number EF189721, was thus substituted with the complete insert with the Accession number KF192265.

Cell-free culture filtrate and RNA sample preparation

Bacterial strains were grown in Improved Minimal Medium (IMM) (Surico *et al*., 1998) at 28 °C in still culture. Time-course analysis of *P. corrugata* CFBP 5454 was determined by sampling aliquots from still cultures incubated in IMM at 28 °C from triplicate flasks at 17, 24, 48, 72 and 96 hpi. To obtain cell-free culture filtrates after centrifugation (9000 × ***g***, 20 min), the supernatant was passed through a 0.22-μm Millipore filter (Millipore, Billerica, MA, USA). Aliquots of all samples were dried and resuspended in sterile water to obtain a 10 × culture filtrate.

Total RNAs were prepared from *P. corrugata* CFBP 5454 and mutant strain cell aliquots, using the RNeasy Mini kit (Qiagen Inc.) according to the manufacturer's directions.

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

The central parts of *crpD* and *crpC* genes were amplified by PCR as 486-bp and 1234-bp fragments, respectively, using the primers MFPkn-fw/rev and NRPSkn-fw/rev (Table 1). These fragments were first cloned into pCR2.1 vector (Invitrogen, Milan, Italy) according to the manufacturer's instructions, and then subcloned by *Bam*HI/*Xba*I digestions into pKnock-Km suicide vector (Alexeyev, 1999), generating PKMMFP and PKMNRPS, respectively. These latter plasmids were transferred into *P. corrugata* CFBP 5454 by triparental mating, generating PCOMFP and PCONRPS, respectively. Transformants were selected on LB agar plates supplemented with kanamycin (100 μg/mL) and confirmed by Southern blot analysis. PCOMFP mutant strain was complemented by introducing pBBR1MCS-5 containing the full-length *P. corrugata crpDE* genes (3224 bp). The sequence was first amplified by PCR using oligonucleotides ABC-fw/rev (Table 1), cloned in pGEM-T easy vector (Promega, Madison, WI, USA) and removed as a *Hind*III/*Bam*HI fragment. The insert was then cloned in the corresponding sites in pBBR1MCS-5 (Kovach *et al*., 1995), yielding pBBR-CrpDE.

Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR analysis was performed using two sets of specific primers, PCR1-fw/rev (523 bp) and PCR2-fw/rev (512 bp) (Table 1), to identify the putative transcripts overlapping the *crpC-crpD* and *crpD-crpE* regions, respectively. Following a DNAse purification step by DNAse I (Invitrogen), 1 μg of RNA was used for cDNA synthesis with Superscript III (Invitrogen) according to the manufacturer's protocol. Genomic DNA was used to test the fidelity of the primer pairs, whereas samples in which reverse transcriptase was not added were used as negative controls. PCRs were performed using a Gene Amp PCR system 9700 (PE Applied Biosystem, Milan, Italy) under the following conditions: an initial 94 °C for 2 min; followed by 35 cycles of 94 °C for 45 s, 56 °C for 45 s and 72 °C for 45 s; and a final extension of 72 °C for 5 min. The RT-PCR products were subjected to electrophoresis with a 1.5% agarose gel.

Transcriptional analysis by Q-PCR

To determine whether *crpC* and *crpD* expression is regulated by the transcriptional regulators PcoR and RfiA, *P. corrugata* CFBP 5454 and derivative mutants GL2 and GLRFIA were analysed by Q-PCR. The same technique was also used to determine *crpC* and *crpD* transcript levels in PCOMFP and PCONRPS strains. The primers for Q-PCR are listed in Table 1 (Abc1 fw/rev for *crpD* and Nrps fw/rev for *crpC*). Reactions were conducted with the Bio-Rad (Bio-Rad, Hercules, CA,USA) iQ5 Cycler and the SYBR GreenER qPCR Super Mix iCycler (Invitrogen), according to the manufacturers' protocols. The *P. corrugata* 16S rRNA (Conte *et al*., 2006) gene was used as housekeeping gene. The relative expression was determined using the comparative CT (cycle threshold) method, also known as the ΔΔCT method (Livak and Schmittgen, 2001). Q-PCR analysis was performed in duplicate on three independent RNA isolations.

In vitro bioassay for AHL and CLP production

Pseudomonas corrugata CFBP 5454 and respective mutant culture filtrates were used to asses AHL and CLP production. Culture filtrates of *P. corrugata* CFBP 5454 were added to the AHL CV026 biosensor to determine AHL production by visual detection of violacein formation (Martinelli *et al*., 2004). Antimicrobial activity was assessed by well-diffusion assay in plates containing a double layer of solidified potato dextrose agar (PDA) (Oxoid) containing the two CLP-sensitive bioindicator strains *R. pilimanae* ATTC26432 and *B. megaterium* ITM100 (Licciardello *et al*., 2009). The region around the well in which growth was prevented was measured. All tests were carried out at least twice in triplicate each time. The presence of CLP in *P. corrugata* CFBP 5454 and derivative mutant cell-free culture filtrates was detected by MALDI-TOF-MS analysis according to Licciardello *et al*. (2012).

Plant inoculations

Pseudomonas corrugata CFBP 5454 and derivative mutants were tested for pathogenicity on tomato cv. Marmande 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). At various time points (3 and 7 dpi), 1 cm of stem was cut either at the inoculation site or at different distances above and below the inoculation site and homogenized in 1 mL of distilled water; serial 10-fold dilutions were plated onto NDA plates with appropriate antibiotics for the selection of the strains using a spiral plater (Eddy Jet, IUL,S.A. Barcelona, Spain). At each sampling time, six plants per strain were analysed. 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 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. Bacterial populations were estimated daily for 4 days in a single leaf disc cut with a boring tool (inner diameter, 0.7 cm) from three individual lesions from three different leaves (Licciardello *et al*., 2007).

Statistical analysis

Data were analysed by one- or three-way ANOVA with CosTat software (CoHort Software, Berkeley, CA, USA). Mean values were compared using Student–Newman–Keuls test.

ACKNOWLEDGEMENTS

This work was funded in part by Ministero dell'Istruzione, dell'Università e della Ricerca, Programma Operativo Nazionale 'Ricerca e Competitività' 2007–2013 (MIUR, PON R&C2007-2013), project 'PolyBioPlast—Technologies and Processes for the Production of Diversely Functionalized Sheets based on Microbial Biopolymers and Biosurfactants (PON01_1377)'.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1 Characteristics of CrpE homologous ABC transporters in plant-associated *Pseudomonas*.