Coregulation of the cyclic lipopeptides orfamide and sessilin in the biocontrol strain *Pseudomonas* sp. CMR12a

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Abstract
Cyclic lipopeptides (CLPs) are synthesized by nonribosomal peptide synthetases (NRPS), which are often flanked by LuxR-type transcriptional regulators. *Pseudomonas* sp. CMR12a, an effective biocontrol strain, produces two different classes of CLPs namely sessilins and orfamides. The orfamide biosynthesis gene cluster is flanked up- and downstream by LuxR-type regulatory genes designated *ofaR1* and *ofaR2*, respectively, whereas the sessilin biosynthesis gene cluster has one LuxR-type regulatory gene which is situated upstream of the cluster and is designated *sesR*. Our study investigated the role of these three regulators in the biosynthesis of orfamides and sessilins. Phylogenetic analyses positioned *OfaR1* and *OfaR2* with LuxR regulatory proteins of similar orfamide-producing *Pseudomonas* strains and the *SesR* with that of the tolaasin producer, *Pseudomonas tolaasii*. LC-ESI-MS analyses revealed that sessilins and orfamides are coproduced and that production starts in the late exponential phase. However, sessilins are secreted earlier and in large amounts, while orfamides are predominantly retained in the cell. Deletion mutants in *ofaR1* and *ofaR2* lost the capacity to produce both orfamides and sessilins, whereas the *sesR* mutant showed no clear phenotype. Additionally, RT-PCR analysis showed that in the sessilin cluster, a mutation in either *ofaR1* or *ofaR2* led to weaker transcripts of the biosynthesis genes, ses-ABC, and putative transporter genes, macA1B1. In the orfamide cluster, mainly the biosynthesis genes *ofaBC* were affected, while the first biosynthesis gene *ofaA* and putative macA2B2 transport genes were still transcribed. A mutation in either *ofaR1*, *ofaR2*, or *sesR* genes did not abolish the transcription of any of the other two.

**KEYWORDS**
cyclic lipopeptides, LuxR, *Pseudomonas*, transcriptional regulators

1 | INTRODUCTION

Cyclic lipopeptides (CLPs) are bacterial metabolites with biosurfactant activity composed of a cyclic oligopeptide lactone ring coupled to a fatty acid tail. The biosynthesis of CLPs is driven by nonribosomal peptide synthetases (NRPS), which are encoded by large gene clusters (Raaijmakers, de Bruijn, & de Kock, 2006). CLPs have drawn increasing interest for their versatile functions in plant beneficial *Pseudomonas*, which include involvement in biofilm formation, motility, and antimicrobial activity against a wide range of microorganisms including fungi,
bacteria, viruses, and oomycetes (reviewed by Olorunleke, Kieu, & Höfte, 2015). Within the different CLP families, several CLP biosynthesis gene clusters have been fully characterized including orfamide, viscosin, massetolide, putisolvin, xantholysin, entolysin, and poaeamide, tolaasin, syringomycin, and syringopeptin, WLIP, arthrotfactin, banamamide, thanapeptin, nunamycin, and nunapeptin (Daes et al., 2014; De Bruijn et al., 2007; De Bruijn, de Kock, de Waard, van Beek, & Raaijmakers, 2008; Dubern, Copposole, Stiekema, & Bloemberg, 2008; Li et al., 2013; Vallet-Gely et al., 2010; Zachow et al., 2015; Scherlach et al., 2013; Wang, Lu, Records, & Gross, 2006; Rotkin-Zadeh et al., 2012; Washio, Lim, Roongsawang, & Morikawa, 2010; Nguyen et al., 2016; Van Der Voort et al., 2015; Michelsen et al., 2015).

The LuxR superfamily consists of transcriptional regulators that contain a DNA-binding helix–turn–helix (HTH) motif in the C-terminal region (Fuqua, Winans, & Greenberg, 1996). In this superfamily, four subfamilies can be distinguished based on domain architecture and the mechanism of regulatory activation. LuxR-like proteins SalA, SyrF, and SyrG are a part of the fourth subfamily, which is characterized by the lack of any defined N-terminal domain. These proteins have been associated with the regulation of the CLPs syringomycin and syringopeptin in Pseudomonas syringae pv. syringae, a plant pathogenic bacterium (Vaughn & Gross, 2016). In various other Pseudomonas species and strains, regulatory genes encoding similar LuxR-like proteins are positioned up- and downstream of the CLP biosynthesis genes (De Bruijn & Raaijmakers, 2009a). Within several CLP families, the regulation of CLP biosynthesis has been attributed to LuxR-type regulators including PsOR (putisolvin) in P. putida (Dubern et al., 2008), ViscA and ViscBC (viscosin) in P. fluorescens SBW25 (De Bruijn & Raaijmakers, 2009a), MassA and MassBC (massetolide) in P. fluorescens SS101 (De Bruijn & Raaijmakers, 2009b), ArfF (arthrotfactin) in P. fluorescens MIS38 (Washio et al., 2010), EtiR (entolysin) in P. entomophila L48T (Vallet-Gely et al., 2010), WlpR (WLIP) in P. putida RW1052 (Rotkin-Zadeh et al., 2012), XtIR (xantholysin) in P. putida BW11M1 (Li et al., 2013), and PcoR and RfaC (corpeptin) in P. corrugata CFBP 5454 (Strano et al., 2015).

In several Pseudomonas strains, the principal regulator of CLP biosynthesis is the GacA/GacS two-component system since a mutation in one of both encoding genes leads to a loss in CLP production (De Bruijn & Raaijmakers, 2009a). The GacA/GacS system is known to activate small RNAs that bind to and sequester translational repressor proteins, which block the ribosomal binding sites in the mRNA of Gac-regulated genes. Two small RNAs (sRNAs) and two repressor proteins, RsmA and RsmE, have been linked to the regulation of entolysin (Vallet-Gely et al., 2010) and massetolide A biosynthesis (Song, Voort, et al., 2015). In the massetolide producer P. fluorescens SS101, these repressor proteins most likely block translation of the LuxR-type transcriptional regulator, MassAR (Song, Voort, et al., 2015), by binding to a specific site called the GacA box. This site comprises a nontranslated leader sequence upstream of the AUG codon on the messenger RNA. In several CLP-producing Pseudomonas strains, a GacA box is present upstream the LuxR regulators flanking the CLP biosynthesis gene cluster suggesting that other CLP-producing Pseudomonas strains may show a similar regulation of lipopeptide biosynthesis (Song, Voort, et al., 2015).

Besides the GacA/GacS regulatory system, N-acylhomoserine lactone (N-AHL)-mediated quorum sensing was shown to be required for viscosin and putisolvin biosynthesis (Cui, Harling, Much, & Darling, 2005; Dubern, Lugtenberg, & Bloemberg, 2006) in P. fluorescens strain 5064 and P. putida strain PCL1445, although this is not the case in certain other Pseudomonas strains (De Bruijn et al., 2008; Dumenyo, Mukherjee, Chun, & Chatterjee, 1998; Kinscherf & Willis, 1999). In P. putida strain PCL1445, two heat shock proteins DnaK and DnaJ located downstream of the Gac system were shown to regulate putisolvin biosynthesis (Dubern, Lugtenberg, & Bloemberg, 2005). Recent studies on the genetic regulation of massetolide A biosynthesis in P. fluorescens SS101 revealed that the serine protease Clp together with the chaperone ClpA regulates the biosynthesis of massetolides via a specific pathway involving the LuxR regulator (MassABC), the heat shock proteins DnaK and DnaJ, and proteins of the tricarboxylic acid (TCA) cycle (De Bruijn & Raaijmakers, 2009b; Song, Aundy, van de Mortel, & Raaijmakers, 2014; Song, Sundqvist, et al., 2015).

Pseudomonas sp. CMR12a is a biocontrol strain isolated from the cocoyam rhizosphere in Cameroon (Perneel et al., 2007). This strain produces two classes of CLPs namely orfamides and sessilins together with two types of phenazines, phenazine-1-carboxylate (PCA) and phenazine-1-carboxamide (PCN) (Daes et al., 2014; Perneel et al., 2007). Orfamides are also produced by biocontrol agents belonging to the P. protegens group (Gross et al., 2007; Jang et al., 2013; Ma, Geudens, et al., 2016; Takeuchi, Noda, & Someya, 2014), while sessilins are structurally related to the tolaasins produced by the mushroom pathogen, P. tolaasii. Sessilins are important for biofilm formation, while orfamides are crucial for the swarming motility of CMR12a (Daes et al., 2014) and both CLPs are important for biocontrol (Daes et al., 2011; Hua & Höfte, 2015; Ma, Hua, Ongenaa, & Höfte, 2016; Olorunleke, Hua, Kieu, Ma, & Höfte, 2015).

In CMR12a, sessilin biosynthesis is governed by three linked NRPS genes namely sesA, sesB, and sesC (Figure 1a) (Daes et al., 2014). These genes are flanked upstream by a nodF-like gene designated sesT, and downstream by macA1 and macB1 genes, which are probably involved in sessilin secretion. MacA and MacB are part of a tripartite secretion system involving an inner membrane protein (MacB), a periplasmic adaptor protein (MacA), and an outer membrane protein (NodT). Similar to sessilin, orfamide biosynthesis is governed by three linked NRPS genes namely ofaA, ofaB, and ofaC (Figure 1b) (Daes et al., 2014). MacA- and macB-like genes putatively involved in orfamide secretion are located downstream of ofaC. Intriguingly, there is no nodF-like gene in the orfamide gene cluster of Pseudomonas sp. CMR12a, while this gene is present in the orfamide gene clusters of P. protegens isolates (Ma, Geudens, et al., 2016). In addition, a LuxR-type regulatory gene, ofaR1, is located upstream of the orfamide biosynthesis cluster and a second one, ofaR2, is situated downstream of the macA2B2 genes, whereas a single LuxR-type regulatory gene, sesR, is located upstream of the sessilin biosynthesis cluster next to the sesT gene (Daes et al., 2014).

In this study, we hypothesized that in Pseudomonas sp. CMR12a, OfaR1 and OfaR2 regulate the biosynthesis of orfamides, whereas
SesR is vital for sessilin biosynthesis. To test our hypothesis, site-directed mutagenesis of the corresponding genes was conducted followed by biochemical and transcriptional analyses.

2 MATERIALS AND METHODS

2.1 Bacterial strains and culture conditions

Bacterial strains, plasmids, and primers used in this study are listed in Table 1. Pseudomonas sp. CMR12a was cultured on Luria–Bertani (LB) agar plates or in liquid LB broth at 28°C. All molecular techniques were performed using standard protocols (Sambrook, Frithsch, & Maniatis, 1989). Escherichia coli strains were grown on LB agar plates or LB broth amended with appropriate antibiotics. Saccharomyces cerevisiae InvSc1 was cultivated on yeast extract–peptone–dextrose (YPD) (Shanks, Caiazza, Hinsa, Toutain, & O'Toole, 2006). Escherichia coli strain WM3064 was used as a host for the plasmids used in site-directed mutagenesis.

2.2 Analysis of CLP production

For LC-ESI-MS analyses, bacterial strains were grown at 28°C in six-well plates with 2.5 ml LB broth per well. Cultures were maintained for variable time periods after which 1 ml of each was centrifuged at 18,900 g for 4 min. Filter-sterilized supernatants were subjected to reverse-phase LC-ESI-MS as described by D’aes et al. (2014). Cells obtained after the centrifugation step were washed once with sterile distilled water resuspended in 1 ml of acetonitrile solution (50%) after which sonication was carried out for 30 s. Following centrifugation, the cell supernatant was filter sterilized and subjected to LC-ESI-MS analysis. Data generated from supernatant and cell analyses were processed to either extract chromatograms or obtain the relative production of sessilins and orfamides using the MassLynx V4.1 software.

2.3 Site-directed mutagenesis

Site-directed mutagenesis of the ofaR1, ofaR2, and sesR genes was performed based on methods described previously (D’aes et al., 2014). To construct each mutant, a fragment of the corresponding LuxR biosynthesis gene was deleted by allelic replacement with vector pMQ30 (Shanks et al., 2006). Primers used for polymerase chain reaction (PCR) and plasmids are described in Table 1. To obtain a deletion plasmid, two coding regions of each LuxR gene were amplified by PCR and these products were cloned next to each other by homologous recombination in S. cerevisiae InvSc1. This plasmid was mobilized into CMR12a by conjugation with E. coli WM3064 and selection on gentamycin. Subsequently, transconjugants that had lost the plasmid during the second crossover event were selected on LB with 10% sucrose after which gene deletion was confirmed by PCR and sequencing (LGC Genomics, Germany).

2.4 Construction of pME6032-based vectors for complementation

A fragment containing the luxR gene was obtained by PCR with specific primers (Table 1). These PCR products were subsequently cloned in the expression vector pME6032 comprising the pTac promoter. The plasmids obtained, pME6032-OfaR1, pME6032-OfaR2, and pME6032-SesR were transformed into E. coli WM3064 via heat shock after which transformed colonies were selected on LB agar plates supplemented with tetracycline 50 μg/ml. Correct integration of fragments
was verified by PCR analysis, restriction analysis of isolated plasmids, and sequencing. These three pME6032-based E. coli WM3064 vectors were transformed into the corresponding Pseudomonas sp. CMR12a LuxR mutants by conjugation. Transformed cells were selected on LB supplemented with 100 μg/ml tetracycline and the presence of pME6032-OfaR1, pME6032-OfaR2, or pME6032-SesR was confirmed by PCR analysis using primers specific for pME6032 and the corresponding luxR gene.

### 2.5 White line-in-agar and swarming motility assays

The white line-in-agar test (Rokni-Zadeh, Li, Yilma, Sanchez-Rodriguez, & De Mot, 2013) was performed in triplicate on Kings’ B (KB) medium. Bacterial strains were cultured in LB broth for 16 hr and washed twice with saline solution (0.85%). The line of bacterial indicator strain (P. protegens Pf-5) in the middle of the plates was made from three drops
(5 μl per drop) of the suspension. Subsequently, 5 μl suspension of each test bacterial strain was spotted at both sides of the line within a 1-cm distance. White precipitate formation in the agar was evaluated after 3 days of growth at 28°C.

For swarming motility assays, 5 μl suspension of each test strain was spotted in the center of LB plates comprising 0.6% agar, left to dry briefly and incubated at 28°C for 24 hr (D’aes et al., 2014). At least two replicates per strain were included, and experiments were repeated at least twice.

2.6 | RNA extraction and reverse transcription-PCR (RT-PCR)

Bacterial cells were grown in still cultures using a six-well plate containing 2.5 ml LB broth per well at 28°C. At 24 hr, growth of strains was determined by measuring optical density OD₆₂₀ of 100 μl in a 96-well plate using a Bio-Rad 680 microplate reader after which 1 ml of cell culture was collected and spun down. Cells were frozen in liquid N₂ and stored at −80°C. For the RNA extraction and complementary DNA (cDNA) synthesis, two biological replicates were used. RNA was isolated from frozen bacterial cells using the Trizol reagent (Sigma), followed by genomic DNA removal using the Turbo DNA-free kit (Ambion/Applied Biosystems). cDNA was synthesized by using the GoScript Reverse Transcription System (Promega). cDNA with RNA equivalent of 100–200 ng was subjected to PCR with specific primers listed in Table S1. The thermal profile used consisted of an initial denaturation step at 95°C for 2 min, followed by 30 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 1 min. The primer pairs were used to amplify cDNA obtained from transcripts corresponding to genes of the sessilin and orfamide biosynthesis gene clusters and their flanking genes including the sesT, sesR, ofaR1, ofaR2, ofaABC, sesABC, and the macAB genes. Transcripts covering adjacent gene pairs of the aforementioned genes were also amplified.

2.7 | Bioinformatic analyses

LuxR-like protein sequences for Pseudomonas sp. CMR12a were obtained from the nucleotide sequences of the sessilin and orfamide biosynthesis gene clusters with GenBank accession numbers JQ309920 and JQ309921, respectively. Other amino acid sequences used for phylogenetic analyses were collected from the National Centre for Biotechnology Information (NCBI) website. Characteristics of strains and protein sequences used in the phylogenetic analyses of LuxR proteins are presented in Table S2. Sequence alignments were made using Muscle (Edgar, 2004) via the software package MEGA6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). Phylogenetic tree was inferred by maximum likelihood (ML) using 1000 bootstrap replicates and was rooted with the LuxR (quorum sensing protein) from Vibrio fischeri. Proteins of N-acyl-l-homoserine lactones (acyl-HSLs)-binding regulators of CMR12a, CmrR and PhzR (De Maeyer, D’aes, Hua, Nam, & Höfte, 2013), were included in this analysis.

Furthermore, bioinformatic tools were employed to check for the presence of Rsm binding sites upstream of the ofaR1, ofaR2, and sesR genes. The query search was conducted using the conserved motif 5′-A/U CANGGAN/A-3′, where N denotes any nucleotide (Song, Voort, et al., 2015). Subsequently, similar nontranslated leader sequences flanking the LuxR transcriptional regulators of several CLP-producing Pseudomonas strains were aligned with the three LuxR regulators of Pseudomonas sp. CMR12a.

3 | RESULTS

3.1 | Growth and production of sessilins and orfamides by CMR12a in shaken and still LB broth cultures

To quantify the production of sessilins and orfamides by CMR12a in shaking (150 rpm) and still LB broth culture conditions, filter-sterilized supernatants and cells were collected at various time points, prepared and subjected to LC-ESI-MS analysis. Time points chosen—17, 20, 24, and 41 hr—corresponded to the late exponential growth phase, early stationary growth phase, stationary growth phase, and death phase of CMR12a. Analyses of relative CLP production (relative peak area/OD₆₂₀) showed that in both culture conditions, coproduction of sessilins and orfamides started at 17 hr (Figure 2). Most of the sessilins produced were immediately secreted into the supernatant, while lower amounts were kept inside (Figure 2a and b). In contrast, orfamides were mainly retained in the cells (Figure 2c). Unlike sessilin secretion, the secretion of orfamides into the LB broth occurred 7 hr after the start of CLP production in both culture conditions (Figure 2d). Aerated cultures reached a higher biomass than still cultures (Figure 2e). In general, aeration had no strong effect on CLP production, although at 24 hr it seemed that more CLPs were retained inside the cell in still conditions.

3.2 | Functional analysis of luxR-type regulatory genes in sessilins and orfamides biosynthesis

LC-ESI-MS analysis revealed the complete abolishment of orfamide and sessilin production in the ofaR1 and ofaR2 mutants (Figure 3a). However, the mutant in the sesR gene, located upstream of the sessilin biosynthesis cluster, still produced sessilins and orfamides.

Additionally, quantitative measurements (relative peak area/OD₆₂₀) of the two CLPs did not reveal any difference between CMR12a and CMR12a–ΔsesR (data not shown). Restored sessilin and orfamide production was observed in the complemented ofaR1 mutant, but not in the complemented ofaR2 mutant (Figure 3a).

Previous results showed that orfamides are important in the swarming motility of CMR12a and that sessilins and orfamides interact to give a white line on KB medium (D’aes et al., 2014). In order to ascertain the cessation of sessilin and orfamide production by the LuxR mutants of CMR12a, swarming motility and white line tests were conducted. Similar to CMR12a, the sesR mutant swarmed on 0.6% LB agar. However, ofaR1 and ofaR2 mutants did not exhibit swarming
motility (Figure 3b). Complementation of the mutants with each of the corresponding target genes cloned into the stable vector pME6032 restored swarming motility in the ofaR1 mutant, but not in the ofaR2 mutant. The white line-in-agar formation is typical for CMR12a when it interacts with an orfamide producer such as P. protegens Pf-5 and is indicative for sessilin production. In our study, CMR12a-ΔofaR1,
CMR12a-ΔorfR2, and the complemented ofaR2 mutants no longer secrete sessilins, since they did not give the white line-in-agar interaction when challenged with the orfamide producer, *P. protegens* Pf-5. The white line-in-agar phenotype was observed, however, for CMR12a, CMR12a-ΔsesR, and the complemented ofaR1 mutant strains (Figure 3b).
3.3 | Transcriptional analysis of flanking and CLP biosynthesis genes in CMR12a and LuxR mutants

Figure 1a and b show primer positions for RT-PCR on the sessilin and orfamide gene clusters, respectively. In order to investigate the transcriptional analysis for ofaABC, sesABC, and their flanking genes, RT-PCR was conducted for CMR12a and LuxR mutants using bacterial cell cultures which were grown in still LB cultures for 24 hr in two replicates (Figure 4a-c).

For the sessilin biosynthetic gene cluster, RT-PCR analysis of the WT strain revealed the transcription of sesA, sesB, and flanking genes, sesT, sesR, macA1, and macB1, whereas sesC was not transcribed. Additionally, the coexpression of sesT-sesR, sesA-sesB, sesB-sesC, sesC-macA1, and macA1-macB1 gene combinations indicate that the sesT-sesR genes on one hand and the sesABC together with macA1B1 genes on the other hand are transcribed from a polycistronic operon (Figure 4a).

In contrast, analysis of the CMR12a-ΔofaR1 mutant mainly revealed the transcription of sesT-sesR and macA1B1 genes. Furthermore, this mutant was characterized by the presence of weak sesAB transcripts. For the CMR12a-ΔofaR2 mutant, similar transcription results as the CMR12a-ΔofaR1 mutant were obtained. Additionally, RT-PCR analyses of the CMR12a-ΔsesR mutant revealed similar results as the WT except for the expected absence of sesT-sesR and sesR expression.

Transcriptional analyses of the orfamide biosynthetic gene cluster were also conducted after growing bacterial cultures for 24 hr. In the WT strain, ofaR1, ofA, ofB, ofC, ofaR2, and the gene combinations of ofaB-ofaC were clearly transcribed, whereas ofaA-ofaB gave a weak transcript (Figure 4b). Additionally, the transcription of macA2 and macB2 and gene combinations of ofaC-macA2 and macA2-macB2 show that ofaABC and macA2B2 are also transcribed from a polycistronic operon. For the sesR mutant, expression and coexpression analyses of all genes and gene combinations showed similar results with CMR12a. In contrast, the CMR12a-ΔofaR1 mutant showed the transcription of ofaA, macA2B2 and ofaR2 coupled with weak ofaB, and ofaB-ofaC transcripts. More so, CMR12a-ΔofaR2 mutant only showed ofaR1, ofA, macA2B2, and weak ofaBC transcripts (Figure 4b).

Furthermore, a mutation in either of the three LuxR-type genes of CMR12a did not appear to abolish the transcription of the other (Figure 4A and B). OfaR1 and ofaR2 mutants appeared to show a weaker transcription of the sesD (syrD-like) gene, whereas the sesR mutant showed similar results with CMR12a (Figure 4c).

3.4 | Phylogenetic analyses of LuxR-type regulatory proteins associated with CLP gene clusters

Phylogenetic analyses of the CLP cluster-associated LuxR-type proteins of CMR12a together with that of other Pseudomonas strains, showed several distinct clusters (Figure 5) as follows: OfaR1 and SesR proteins clustered together with other LuxR-type regulators located upstream of CLP biosynthesis genes. Similarly, OfaR2 clustered with LuxR-type regulators located downstream of the CLP biosynthesis genes. Specifically, SesR clustered with other LuxR-type regulators within the tolasin group, while OfaR1 and OfaR2 clustered with regulators which flank orfamide-coding genes in other Pseudomonas strains including P. protegens Pf-5 (Loper & Gross, 2007). The AHL-binding regulators of CMR12a, CmrR and PhzR, formed a separate cluster together with the LuxR of V. fischeri indicating that they belong to a separate subfamily of regulators (Figure 5).

3.5 | Presence of Rsm binding sites upstream of LuxR transcriptional regulators

Genomic search for putative Rsm binding sites was conducted within the sequences upstream of the three LuxR regulatory genes of CMR12a. Conserved GGA motifs upstream of the ATG start codon could be identified. Sequence alignment of these sequences with their homologs in CLP-producing Pseudomonas strains showed the similarity of these regions upstream of sessilins and orfamide biosynthetic gene clusters with those of previously described CLPs (Figure 6).
**FIGURE 5** Phylogenetic analysis of the LuxR-type regulators flanking the orfamide and sessilin biosynthesis genes of *Pseudomonas* sp. CMR12a (highlighted in gray). Also included in this analysis are the LuxR-type regulators of other *Pseudomonas* CLP biosynthesis genes, and AHL-binding regulators LuxR from *Vibrio fischeri*, and PhzR and CmrR from *Pseudomonas* sp. CMR12a. The dendrogram was generated by maximum likelihood using 1,000 resampled datasets. Percentage bootstrap values are indicated at branching nodes while the bar indicates sequence divergence.

**FIGURE 6** Alignment of the regions upstream of the LuxR transcriptional regulatory genes which flank different lipopeptide biosynthesis gene clusters including *Pseudomonas* *fluorescens* SS101, *Pseudomonas* sp. MIS38, *P. fluorescens* P0-1, *P. protegens* Pf-5, *P. putida* PCL1445, *P. entomophila* L48T, *P. syringae* pv. tomato DC3000, and our study strain *Pseudomonas* sp. CMR12a. The conserved GGA motif is highlighted in red. The translation initiation ATG codon is indicated at the 3′ end, while * indicates the sequences of the test strain used in this study.
Our study revealed that the LuxR-like transcriptional regulators, OfaR1 and OfaR2, which are associated with the orfamide gene cluster not only regulate orfamide biosynthesis but also sessilin biosynthesis, while we could not find a clear function for the LuxR-like regulator, SesR, associated with the sessilin gene cluster.

LC-ESI-MS analysis revealed that orfamide and sessilin production commences concurrently in the late exponential phase, but orfamide is mainly retained inside the cell and secreted much later and in lower amounts than sessilin. We have previously shown that the release of orfamide in the environment is hampered by sessilin and hypothesized that both compounds compete for the same outer membrane efflux transporter, SesT (D’aes et al., 2014). Here, we show that the sesT gene, located upstream of the sessilin biosynthetic cluster, is expressed from an operon together with sesR. A mutation in sesR, however, does not seem to affect CLP production or secretion. We are currently investigating the secretion of orfamides and sessilins in more detail by mutant analysis of putative transport genes including macAB, sesT, and sesD. In contrast, ofaR1 and ofaR2 mutants completely lost the capacity to produce both sessilins and orfamides as evidenced by the absence of swelling, lack of a white line-in-agar phenotype, and confirmed by LC-ESI-MS analysis. Also, in the biocontrol strain P. fluorescens SBW25, mutations in the LuxR-type regulatory genes viscAR and viscBCR, located up- and downstream of the viscosin biosynthesis cluster, led to a loss of viscosin production (De Bruijn & Raaijmakers, 2009a). Other homologs of ofaR1, located upstream of their NRPS genes, have been shown to be necessary for the production of putisolvin (psOR) (Dubern et al., 2008), arthrofactin (arfF) (Washio et al., 2010), and entolysin (etIR) (Vallet-Gely et al., 2010).

So far, coregulation of different classes of CLPs in the same strain has only been demonstrated for plant pathogenic Pseudomonas bacteria. In the bean pathogen P. syringae pv. syringae B728a, three LuxR-like proteins, SalA, SyrF, and SyrG, were shown to control the biosynthesis of the CLPs syringopeptin and syringomycin (Vaughn & Gross, 2016). SalA controls the expression of both syrG and syrF (Lu, Scholz-Schroeder, & Gross, 2002). Furthermore, qRT-PCR analysis of deletion mutants in syrF and syrG showed that both genes require a functional salA gene for activation. In addition, SyrG appears to function as an upstream transcriptional activator of syrF (Vaughn & Gross, 2016). The situation in Pseudomonas sp. CMR12a is different since a mutation in either ofaR1, ofaR2, or sesR did not abolish the transcription of the other, although the transcript of ofaR1 may seem weaker in the ofaR2 mutant. Our method does not allow precise transcript quantification and further investigation by quantitative RT-PCR is needed. Likewise in the viscosin producing strain—P. fluorescens SBW25, a mutation in either viscAR or viscBCR, luxR genes located up- and downstream of the viscABC biosynthesis genes, did not substantially affect the transcription of the other (De Bruijn & Raaijmakers, 2009a) indicating that both LuxR regulators do not transcriptionally affect each other.

Transcriptional analyses showed that for both the sessilin and orfamide gene clusters, their biosynthesis genes, sesABC and ofaABC, together with putative transport genes, macAB, are most likely transcribed from a polycistronic operon, which is probably regulated by OfaR1 and OfaR2. The absence of a sessC transcript in CMR12a could be due to primer specificity problems since a coexpression was observed for sesB-sesc and sesC-macA1. With respect to the orfamide gene cluster, worthy of note was the fact that mutants in ofaR1 and ofaR2 still showed clear transcripts for ofaA and macA2B2 genes. These results indicate that besides the single promoter which enables the transcription of ofaABC and macA2B2 genes, separate promoters for ofaA and macA2B2 may be present, which are not controlled by OfaR1 and OfaR2. Unfortunately, little information is available about the gene coexpression of other CLP gene clusters except for WLIP (Rokni-Zadeh et al., 2012), so we could not ascertain if the presence of multiple promoters as was observed in the orfamide gene cluster is a frequent occurrence. In this respect, it is interesting to notice that in beneficial Pseudomonas spp., the genomic region encoding the first CLP biosynthesis gene is often unlinked with the other two biosynthesis genes, which are coexpressed. This is for instance the case for viscosin, massetolide, WLIP, xantholysin, entolysin, and poaeamide (De Bruijn et al., 2007, 2008; Li et al., 2013; Rokni-Zadeh et al., 2012; Vallet-Gely et al., 2010; Zachow et al., 2015).

During this study, we were unable to complement the CMR12a-ΔofaR2 mutant. Considering the fact that the macB2 gene associated with the orfamide gene cluster gave a weaker transcript than macA2 for CMR12a, it is possible that ofaR2 is transcribed from a longer transcript which spans across part of the macB2 gene. This would result in an antisense overlap that could influence the expression of macB2 by transcription attenuation (Sesto, Wurtzel, Archambaud, Sorek, & Cossart, 2012). This obviously requires further investigation.

In our study, phylogenetic analysis of LuxR-type proteins, positioned up- and downstream of the CLP gene clusters of CMR12a together with previously described CLP-associated LuxR regulators revealed that OfaR1 and SesR clustered together with known LuxR-type regulators located upstream of the CLP biosynthesis genes, whereas OfaR2 clustered with those located downstream. LuxR regulators from strains which produce similar CLPs, for example, orfamide producers P. protegens Pf-5 and Pseudomonas sp. CMR12a, cluster together. An exception is the LuxR regulator for poaeamide, P. poae RE*1-1-14 which although shares a structural relationship with orfamide (Zachow et al., 2015), clusters with LuxR regulators of CLPs belonging to the viscosin family. The LuxR regulator (WirP) of the WLIP producer—P. reductans LMG 5329, showed a higher homology with LuxR regulators of the viscosin family compared with that of another WLIP producer—P. putida RW1052 (Rokni-Zadeh et al., 2013). This decreased conservation suggests that the biosynthetic gene cluster of poaeamide might have evolved separately. Our results further indicate that LuxR-type regulators of CMR12a belong to the same subfamily as in other plant beneficial Pseudomonas strains including P. protegens Pf-5, P. fluorescens SS101, and P. fluorescens SBW25, which produce orfamide, massetolide, and viscosin, respectively (De Bruijn & Raaijmakers, 2009a; De Bruijn et al., 2008; Loper & Gross, 2007). Given that LuxR transcriptional regulators of P. syringae pv. syringae cluster with all LuxR regulators analyzed during this study, our results indicate that similar to this plant pathogenic strain, these other LuxR regulators, including OfaR1,
OflaR2, and SesR, belong to the fourth LuxR family which is characterized by the absence of any defined N-terminal domain ( Vaughn & Gross, 2016 ).

During this study, a genomic search and subsequent alignment of sequences upstream of ofaR1, ofaR2, and sesR with their homologs in other lipopeptide biosynthesis genes of Pseudomonas strains, showed that Rsms binding sites were located upstream of all these luxR-like genes of CMR12a. Given the fact that this Rsms binding site, alternatively called the GacA box, was found upstream of multiple CLP biosynthesis genes (Song, Voort, et al., 2015a) in different Pseudomonas strains, our results suggest that the Gac/Rsms-mediated regulation of CLPs might be a general phenomenon in most biocontrol CLP-producing Pseudomonas spp.

In conclusion, this study establishes that sessilin and orfamide production in CMR12a are coregulated by two of the three luxR-type genes namely ofaR1 and ofaR2. Our findings show that either OfaR1 or OfaR2 can regulate the biosynthesis of these two CLPs, while the function of SesR remains unclear.

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CONFLICT OF INTEREST

None declared.

REFERENCES

Cui, X., Harling, R., Mutch, P., & Darling, D. (2005). Identification of N-3-hydroxoyctanoyl-homoserine lactone production in Pseudomonas fluorescens 5064, pathogenic to broccoli, and controlling biosurfactant production by quorum sensing. European Journal of Plant Pathology, 111, 297–308.

D’aes, J., Hua, G. K. H., De Maeyer, K., Pannecoque, J., Forrez, I., Ongena, M., … Höfte, M. (2011). Biological control of Rhizoctonia root rot on bean by phenazine- and cyclic lipopeptide-producing Pseudomonas CMR12a. Phytopathology, 101, 996–1004.

D’aes, J., Kieu, N. P., Lécêbre, V., Tokarski, C., Olorunleke, F. E., De Maeyer, K., … Ongena, M. (2014). To settle or to move? The interplay between two classes of cyclic lipopeptides in the biocontrol strain Pseudomonas CMR12a Environmental Microbiology, 16, 2282–2300.

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

De Bruijn, I., de Kock, M. J. D., Yang, M., de Waard, P., van Beek, T. A., & Raaijmakers, J. M. (2007). Genome-based discovery, structure prediction and functional analysis of cyclic lipopeptide antibiotics in Pseudomonas species. Molecular Microbiology, 63(2), 417–428.

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

De Bruijn, I., & Raaijmakers, J. M. (2009b). Regulation of cyclic lipopeptide biosynthesis in Pseudomonas fluorescens by the CppP Protease. Journal of Bacteriology, 191, 1910–1923.

De Maeyer, K., D’aes, J., Hua, G. K. H., Nam, P. K., & Höfte, M. (2013). N-acetyl homoserine lactone quorum sensing signaling in phenazine and cyclic lipopeptide producing Pseudomonas sp. CMR12a from the red cocoyam rhizosphere. In F. J. De Bruijn (ed.), Molecular microbial ecology of the rhizosphere (pp. 763–774). New Jersey, USA: John Wiley & Sons.

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

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

Dubern, J. F., Lugtenberg, B. J. J., & Bloemberg, G. V. (2006). The ppul-rr-pspR quorum-sensing system regulates biofilm formation of Pseudomonas putida PCL1445 by controlling biosynthesis of the cyclic lipopeptides putisolvin I and II. Journal of Bacteriology, 188, 2898–2906.

Dumenyo, C. K., Mukherjee, A., Chun, W., & Chatterjee, A. K. (1998). Genetic and physiological evidence for the production of N-acetyl homoserine lactones by Pseudomonas syringae pv. syringae and other fluorescent plant pathogenic Pseudomonas species. European Journal of Plant Pathology, 104, 569–582.

Edgar, R. C. (2004). MUSCLE: Multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research, 32, 1792–1797.

Fuqua, C., Winans, S. C., & Greenberg, E. P. (1996). Census and consensus in bacterial ecosystems: The LuxR-LuxI family of quorum-sensing transcriptional regulators. Annual Review of Microbiology, 50, 727–751.

Gross, H., Stockwell, V. O., Henkels, M. D., Nowak-Thompson, B., Loper, J. E., & Gerwick, W. H. (2007). The Genomisotopic Approach: A systematic method to isolate products of orphan biosynthetic gene clusters. Chemistry & Biology, 14, 53–63.

Hanahan, D. (1983). Studies on transformation of Escherichia coli with plasmids. Journal of Molecular Biology, 166, 557–580.

Heeb, S., Blumer, C., & Haas, D. (2002). Regulatory RNA as mediator in GacS/RmsA-dependent global control of exoproduct formation in Pseudomonas fluorescens CHA0. Journal of Bacteriology, 184, 1046–1056.

Hua, G. K. H., & Höfte, M. (2015). The involvement of phenazines and cyclic lipopeptide sessilin in biocontrol of Rhizoctonia root rot on bean (Phaseolus vulgaris) by Pseudomonas sp. CMR12a is influenced by substrate composition. Plant and Soil, 388, 243–253.

Jang, J. Y., Yang, S. Y., Kim, Y. C., Lee, C. W., Park, M. S., Kim, J. C., & Kim, I. S. (2013). Identification of orfamide A as an insecticidal metabolite produced by Pseudomonas protegens F6. Journal of Agricultural and Food Chemistry, 61, 6786–6791.

Kinscherf, T. G., & Willis, D. K. (1999). Swarming of Pseudomonas syringae B728a requires GacS (LemA) and GacA but not the acyl-homoserine lactone biosynthetic gene Ahil. Journal of Bacteriology, 181, 4133–4136.

Li, W., Rotki-Zadeh, H., De Vleeschouwer, M., Ghequire, M. G. K., Sinnaeve, D., Xie, G.-L., … De Mot, R. (2013). The antimicrobial compound xantholysin defines a new group of Pseudomonas cyclic lipopeptides. PLoS ONE, 8, e62946.

Loper, J. E., & Gross, H. (2007). Genomic analysis of antifungal metabolite production by Pseudomonas fluorescens Pf-5. European Journal of Plant Pathology, 119, 265–278.

Lu, S. E., Scholz-Schroeder, B. K., & Gross, D. C. (2002). Characterization of the SaIA, SyrF, and SyrG regulatory genes located at the right border of the syringomycin gene cluster of Pseudomonas syringae pv. syringae. Molecular Plant Microbe Interactions, 15, 43–53.
Ma, Z., Geudens, N., Kieu, N. P., Sinnaeve, D., Ongena, M., Martins, J. C., & Höfte, M. (2016). Biosynthesis, chemical structure, and structure-activity relationship of orfamide lipopeptides produced by Pseudomonas protegens and related species. Frontiers in Microbiology, 7, 382.

Ma, Z., Hua, G. K. H., Ongena, M., & Höfte, M. (2016b). Role of phenazines and cyclic lipopeptides produced by Pseudomonas sp. CMR12a in induced systemic resistance on rice and bean. Environmental Microbiology Reports, 8(S), 896–904.

Michelsen, C. F., Watrous, J., Glaring, M. A., Kersten, R., Koyama, N., Dorrestein, P. C., & Stougaard, P. (2015). Nonribosomal peptides, key biocontrol components for Pseudomonas fluorescens In5, isolated from a Greenlandic suppressive soil. MBio, 6, e00079–15.

Nguyen, D. D., Melnik, A. V., Koyama, N., Lu, X., Schorn, M., Fang, J., ... Dorrestein, P. C. (2016). Indexing the Pseudomonas specialized metabolome enabled the discovery of poaeamide B and the bananamides. Nature Microbiology, 2, 16197.

Olorunleke, F. E., Hua, G. K. H., Kieu, N. P., Ma, Z., & Höfte, M. (2015). Interplay between orfamides, sessilins and phenazines in the control of Rhizoctonia diseases by Pseudomonas sp. CMR12a. Environmental Microbiology Reports, 7, 774–781.

Olorunleke, F. E., Kieu, N. P., & Höfte, M. (2015) Recent advances in Pseudomonas biocontrol. In J. Murillo, B. A. Vinatzer, R. W. Jackson & D. L. Arnold (Eds.), Bacterial-plant interactions: Advance research and future trends (pp. 167–198). Cambridgeshire: Caister Academic Press.

Perneel, M., Heyman, J., Adiobo, A., De Maeyer, K., Raaijmakers, J. M., De Vos, P., & Höfte, M. (2007). Characterization of CMR5c and CMR12a, novel fluorescent Pseudomonas strains from the cocoyam rhizosphere with biocontrol activity. Journal of Applied Microbiology, 103, 1007–1020.

Raaijmakers, J. M., de Bruijn, I., & de Kock, M. J. D. (2006). Cyclic lipopeptide production by plant-associated Pseudomonas spp.: Diversity, activity, biosynthesis, and regulation. Molecular Plant Microbe Interactions, 19, 699–710.

Rokni-Zadeh, H., Li, W., Sanchez-Rodriguez, A., Sinnaeve, D., Rozenks, J., Martins, J. C., & De Mot, R. (2012). Genetic and functional characterization of cyclic lipopeptide WLIP production by rice rhizosphere isolate Pseudomonas putida RW1052. Applied and Environmental Microbiology, 78, 4826–4834.

Rokni-Zadeh, H., Li, W., Yilma, E., Sanchez-Rodriguez, A., & De Mot, R. (2013). Distinct lipopeptide production systems for WLIP (white line-inducing principle) in Pseudomonas fluorescens and Pseudomonas putida. Environmental Microbiology Reports, 5, 160–169.

Saltikov, C. W., & Newman, D. K. (2003). Genetic identification of a respiratory arsenate reductase. Proceedings of the National Academy of Sciences, 100, 10983–10988.

Sambrook, J., Frithsch, E. F., & Maniatis, T. (1989). Molecular cloning: A laboratory manual (2nd ed.). Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Scherlach, K., Lackner, G., Graupner, K., Pidot, S., Bretschneider, T., & Hertweck, C. (2013). Biosynthesis and mass spectrometric imaging of tolaasin, the virulence factor of brown blotch mushroom disease. ChemBioChem, 14, 2439–2443.

Sesto, N., Wurtzel, O., Archambaud, C., Sorek, R., & Cossart, P. (2012). The excluson: A new concept in bacterial antisense RNA-mediated gene regulation. Nature Reviews Microbiology, 11(2), 75–82.

Shanks, R. M. Q., Caiazzo, N. C., Hinsa, S. M., Toutain, C. M., & O’Toole, G. A. (2006). Saccharomyces cerevisiae-based molecular tool kit for manipulation of genes from gram-negative bacteria. Applied and Environmental Microbiology, 72, 5027–5036.

Song, C., Aundy, K., van de Mortel, J., & Raaijmakers, J. M. (2014). Discovery of new regulatory genes of lipopeptide biosynthesis in Pseudomonas fluorescens. FEMS Microbiology Letters, 356, 166–175.

Song, C., Sundqvist, G., Malm, E., de Bruijn, I., Kumar, A., van de Mortel, J., ... Raaijmakers, J. M. (2015b). Lipopeptide biosynthesis in Pseudomonas fluorescens is regulated by the protease complex ClpAP. BMC Microbiology, 15, 29.

Song, C., van der Voort, M., van de Mortel, J., Hassan, K. A., Elbourne, L. D. H., Paulsen, I. T., ... Raaijmakers, J. M. (2015). The Rsm regulon of plant growth-promoting Pseudomonas fluorescens SS101: Role of small RNAs in regulation of lipopeptide biosynthesis. Microbial Biotechnology, 8, 296–310.

Strano, C. P., Bella, P., Liciardiello, G., Fiore, A., Lo Piero, A. R., Fogliano, V., ... Catara, V. (2015). 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. Molecular Plant Pathology, 16, 495–506.

Takeuchi, K., Noda, N., & Someya, N. (2014). Complete genome sequence of the biocontrol strain Pseudomonas protegens Cab57 discovered in Japan reveals strain-specific diversity of this species. PLoS ONE, 9, e93683.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution, 30, 2725–2729.

Vallet-Gely, I., Novikov, A., Augusto, L., Lielh, P., Bolbach, G., Péché-Tarr, M., ... Lemaître, B. (2010). Association of hemolytic activity of Pseudomonas entomophila, a versatile soil bacterium, with cyclic lipopeptide production. Applied and Environment Microbiology, 76, 910–921.

Van Der Voort, M., Meijer, H. J. G., Schmidt, Y., Watrous, J., Dekkers, E., Mendes, R., ... Raaijmakers, J. M. (2015). Genome mining and metabolic profiling of the rhizosphere bacterium Pseudomonas sp. SH-CS2 for antimicrobial compounds. Frontiers in Microbiology, 6, 693.

Vaughn, V. L., & Gross, D. C. (2016). Characterization of SalA, SyrF, and SyrG genes and attendant regulatory networks involved in plant pathogenesis by Pseudomonas syringae pv. syringae B728a. PLoS ONE, 11, e0150234.

Wang, N., Lu, S. E., Records, A. R., & Gross, D. C. (2006). Characterization of the transcriptional activators SalA and SyrF, which are required for syringomycin and syringopeptin production by Pseudomonas syringae pv. syringae. Journal of Bacteriology, 188(9), 3290–3298.

Wasiko, K., Lim, S. P., Roongsawang, N., & Morikawa, M. (2010). Identification and characterization of the genes responsible for the production of the cyclic lipopeptide arthrofactin by Pseudomonas sp. MIS38. Bioscience, Biotechnology, and Biochemistry, 74, 992–999.

Zachow, C., Jahanshah, G., de Bruijn, I., Song, C., Ianni, F., Pataj, Z., ... Raaijmakers, J. M. (2015). The novel lipopeptides poaeamide of the endophyte Pseudomonas poae RE*1-1-14 is involved in pathogen suppression and root colonization. Molecular Plant Microbe Interactions, 28(7), 800–810.

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