Small RNAs regulate the biocontrol property of fluorescent Pseudomonas strain Psd

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A B S T R A C T

The production of biocontrol factors by Pseudomonads is reported to be controlled at the post-transcriptional level by the GacS/GacA signal transduction pathway. This involves RNA-binding translational repressor proteins, RsmA and RsmE, and the small regulatory RNAs (sRNAs) RsmX, RsmY, and RsmZ. While the former represses genes involved in secondary metabolite production, the latter relieves this repression at the end of exponential growth. We have studied the fluorescent Pseudomonas strain Psd, possessing good biocontrol potential, and confirmed the presence of rsmY and rsmZ by PCR amplification. Gene constructs for all the three small RNAs (RsmX, RsmY and RsmZ) carried on broad host-range plasmid, pME6032 were mobilized into strain Psd. Expression analysis of gacA in the recombinant strains over-expressing rsmX (Psd-pME7320), rsmY (Psd-pME6359) and rsmZ (Psd-pME6918) revealed a significant upregulation of the response regulator. Besides, a remarkable down-regulation of rsmA was also reported in all the strains. The variant strains were found to produce comparatively higher levels of phenazines. Indole acetic acid levels were higher to some extent, and strain Psd-pME6918 also showed elevated production of HCN. The tomato seedlings infected with Fusarium oxysporum and Verticillium dahliae in the presence of culture filtrate of the recombinant strains showed better plant protection response in comparison to the wild-type strain Psd. These results suggest that small RNAs are important determinants in regulation of the biocontrol property of strain Psd.

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1. Introduction

Rhizosphere is an ecologically diverse niche, constituting a variety of metabolically versatile microorganisms, both phytopathogenic and phyto-beneficial. The colonization of host-tissues by microbes is based on intricate cellular regulatory networks (Harfouche et al., 2015). Phyto-beneficial bacteria, including Azotobacter, Azospirillum, Bacillus, Burkholderia, Erwinia, Flavobacterium, Pseudomonas, and Serratia promote plant growth by a number of direct and indirect mechanisms. The direct mechanisms include biological nitrogen fixation, production of phytohormones (such as auxins, cytokinins and gibberellins), solubilization of key plant nutrients like iron and phosphorus, and siderophore production (Kochar et al., 2013a,b; Ahemad and Kibret, 2014). On the other hand, indirect mechanisms majorly comprise the biocontrol pathways, including antibiotic production, secretion of fungal-cell wall degrading enzymes, etc. (Bhattacharyya and Jha, 2012; Ahemad and Kibret, 2014).

In a wide variety of Gammaproteobacteria, the production of antibiotics and other secondary metabolites, including hydrogen cyanide (HCN), indole-3-acetic acid (IAA), extracellular proteases and tryptophan side-chain oxidase (TSO) are regulated by a highly-conserved, two-component signal transduction system consisting of a sensor kinase, GacS, and its cytoplasmic cognate response regulator, GacA (Heeb and Haas, 2001; Haas and Keel, 2003; Workentine et al., 2009; Hassan et al., 2010; Wang et al., 2013). While GacS acts as the sensory unit, GacA mediates the required changes in gene expression in response to the signal. Small regulatory RNAs (sRNAs), including CsrB/CsrC (E. coli), RsmB (Erwinia carotovora), and Rsm X/Y/Z (Pseudomonas sp.), play an integral role in the pathway, mediating a number of processes, including secretion of secondary metabolites (such as

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Table 1
Bacterial/fungal strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strain or Plasmid</th>
<th>Relevant characteristics</th>
<th>Reference</th>
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</table>
| **BACTERIAL STRAINS**
| **Pseudomonas strains**
| Psd | Wild-type strain, isolated from V. mungo roots, Tc<sup>c</sup>, Ap<sup>c</sup>, Cm<sup>c</sup> | Dr. A.K. Saxena, IARI, New Delhi, India |
| CHA0 | Standard strain for chlorogluconol (DAPG) & Pyoluteorin (PLT) antibiotics and other PGRP traits | Prof. Dieter Haas, University of Lausanne, Switzerland |
| Psd 30 | Tns5 deleted variant of strain Psd, kan<sup>+</sup> supE44 hisdR17 (r<sub>−</sub> m<sub>−</sub>) recA1 endA1 thi-1 gyrA96 relA1 lac- F'LproAB+ lacIq lacZ ΔDM15 Tn10(Tc<sup>c</sup>) | Lab stock |
| E. coli XL1-Blue | T357 | Lab Stock |
| **Fungal strains**
| Fusarium oxysporum | Plant fungal pathogen causing wilt disease in tomato plants | IARI, New Delhi |
| Verticillium dahliae | Plant fungal pathogen causing wilt disease in tomato plants | IARI, New Delhi |
| **Plasmids** |  |  |
| pME6032 | Expression vector; pVS1-p15A shuttle vector; Tc<sup>c</sup> | Prof. D. Haas, Switzerland |
| pME6359 | Ptac-rsmZ fusion at +1 site in pME6032; Tc<sup>c</sup> | Prof. D. Haas, Switzerland |
| pME6918 | Ptac-rsmY fusion at +1 site in pME6032; Tc<sup>c</sup> | Prof. D. Haas, Switzerland |
| pME7320 | Cloning vector; ColE1 replicon; Ap<sup>c</sup> | Lab Stock |
| pBKs+ | Plasmop DNA carrying 900 bp Km<sup>c</sup> cassette | Lab stock |
| pOT2 | 5.3 kb plasmid containing 538 bp Gm<sup>c</sup> cassette | Lab Stock |

HCN, phenazines, 2,4-diacetylphloroglucinol, pyoluteorin, indole-3-acetic acid, siderophores, etc.) quorum sensing, motility, and virulence (Yu et al., 2014; Zha et al., 2014; Harfouche et al., 2015; Miller et al., 2016). These non-coding RNAs (ncRNAs) possess multiple hairpin loops with a number of exposed trinucleotide GGA motifs (Wang et al., 2013). Another important component of the signal transduction pathway are the RNA-binding proteins, RsmA and RsmE, which belong to the RsmA/CsrA family (Wang et al., 2013). These proteins block the translation of certain target mRNAs, encoding various antifungal metabolites, virulence factors and carbon storage compounds, by recognizing and binding to the 5’-GGA-3’ motifs in these mRNAs (Reimann et al., 2005; Wang et al., 2013). This repression is relieved at the end of the active growth by the sRNAs, RsmX, Y and Z, which scavenge RsmA and RsmE by sequestration, thus leading to their post-transcriptional repression (Haas and Keel, 2003; Valverde et al., 2004; Babitzke and Romeo, 2007). The expression/over-expression of these regulatory RNAs is believed to titrate the translational repressors, thereby freeing the ribosome binding site of target mRNAs, leading to the expression of the required functions (Pessi et al., 2001; Haas and Defago, 2005).

There is a considerable variation in the number of sRNAs across different bacterial species. For instance, in certain bacterial species, like V. cholerae, P. fluorescens, P. protegens and P. syringae, the Gac system induces the transcription of three sRNAs (RsmX, RsmY and RsmZ), while in case of bacteria like P. aeruginosa, E. coli, and S. enterica, only two sRNAs (RsmY and RsmZ) have been reported (Kay et al., 2006; Miller et al., 2016). Despite the functional redundancy between these sRNAs, their numbers and the auxiliary factors regulating their expression vary between different species (Miller et al., 2016). Since fluorescent Pseudomonads, being the dominant members of the plant growth promoting bacteria (PGPB), are involved in the biocontrol of various fungal pathogens by synthesizing allelopathic factors such as toxins, antibiotics, and siderophores (Bhattacharyya and Jha, 2012; Ahemad and Kibret, 2014), it is pertinent to understand the role of sRNAs in regulation of these processes.

The fluorescent Pseudomonas strain Psd, originally isolated from the rhizosphere of Vigna mungo, has been characterized for its multiple plant growth-promoting and biocontrol properties (Upadhyay and Srivastava, 2008, 2010a; Kocher et al., 2011; Kocher et al., 2013a,b; Upadhyay and Srivastava, 2014; Sirohi et al., 2015). Various anti-fungal metabolites are produced by the strain, such as siderophores, HCN, lipodepsipeptide and two broad-host range antibiotics, phenazine-1-carboxylic acid (PCA) and pyrrolnitrin (Upadhyay and Srivastava, 2008, 2010b). Besides, the presence of gacS and gacA in the bacterium indicated towards the presence of two component regulatory system GacS/GacA in regulation of the aforementioned traits (Upadhyay and Srivastava, 2008). The present investigation was targeted to decipher the presence of small regulatory RNA genes in the fluorescent Pseudomonas strain Psd, and to understand their role in biocontrol potential of the bacterium through over-expression analysis.

2. Materials and methods

2.1. Bacterial/fungal strains and culture conditions

The bacterial and fungal strains used in this study were obtained from different sources and are listed in Table 1. Besides wild-type strain Psd, we have also used a mutant of strain Psd, Psd-30, which shows a changed phenotype for many of the secondary metabolites, except for siderophores production. The media used, culture conditions and maintenance of the strains are as described in previous reports (Upadhyay and Srivastava, 2008, 2010b).

2.2. PCR amplification and cloning

Bacterial genomic DNA was isolated using GenElute™ bacterial genomic DNA Kit (Sigma-Aldrich, USA), as per the manufacturer’s instructions. The isolated DNA was used as a template for amplification of rsm genes by PCR. All the genetic manipulations were carried out according to the procedures described earlier (Sambrook et al., 2001). The details of primers used and the respective PCR conditions used are given in Table 2.

The PCR products obtained were eluted, purified and digested with the appropriate restriction enzymes. These fragments were directionally cloned in pBKs(+) and transformed in E. coli XL-1 Blue using standard methods (Sambrook et al., 2001). The frag-
ments were subsequently sequenced at DNA sequencing facility of Department of Biochemistry (University of Delhi South Campus, New Delhi).

2.3. Modification of constructs carrying small regulatory RNA genes for their transfer to strain Psd

Constructs pME7320, pME6918 and pME6359, originally derived from P. protegens CHA0 were a kind gift from Prof. D. Haas, University of Lausanne, Switzerland. These were used as source for genes rsmX, rsmY and rsmZ, respectively. Cloned under P_tac promoter. These plasmids carried Tet’ as the selectable marker. As Psd also carries the same selection marker, it became mandatory to modify the constructs for facilitating the selection of transformants. P. protegens CHA0 strains containing pME7320, pME6918 and pME6359, respectively were grown in LB with 10 μg/ml of Tet and plasmids were isolated by alkaline lysis method (Sambrook et al., 2001). These constructs were modified to carry kan’ or gen’ cassette. The kan’ gene was isolated from pTnMod-OKm3 as SacI fragment through restriction digestion and subsequently cloned into the SacI restricted, pME7320 and pME6359. The gen’ gene was isolated from the plasmid pOT2 by PCR carrying AattI site and placed into pME6918 at the respective site. The constructs so obtained were electro-transformed in strain Psd as described previously (Upadhyay and Srivastava, 2010b). The transformants were selected on respective antibiotics after overnight incubation.

2.4. Sequence analysis of rsmY and rsmZ genes derived from strain Psd

The nucleotide–nucleotide Basic Local Alignment Search Tool (BLASTn) program of NCBI was used to compare the nucleotide sequences originating from strain Psd to the sequences deposited in the database and the statistical significance of matches was derived. This was followed by sequence analysis for conserved GGA motifs.

2.5. Expression analysis

Total RNA was isolated from idio-phase-bacterial cultures with the RNasea Protect Bacteria Mini Kit (Qiagen, The Netherlands), as per manufacturer’s instructions. DNase-free RNA (1 μg) was used in a one-step RT-PCR reaction (RevertAid First Strand cDNA Synthesis Kit, Thermo Scientific, USA) performed with the random hexamer primers, according to manufacturer’s instructions. The cDNA prepared was subjected to qPCR using SYBR GREEN reaction mix in 7900HT Fast Real-Time PCR System (Applied Biosystems, USA). The reaction included initial activation (58 °C for 2 min and 95 °C for 10 min), amplification for 40 cycles (95 °C for 15 s, 60 ° C for 1 min). This was then followed by a gradual increase in temperature from 58 °C to 95 °C for generating the melt curve. The dissociation program was 95 °C for 15 s, 60 °C for 15 s and 95 °C for 15 s. The experiment included three biological replicates and each biological replicate was evaluated by three technical replicates. The constitutively expressed gene of Pseudomonas sp., for 16 s rRNA, was taken as the calibrator in order to normalize gene expression levels. The Ct values obtained in the experiment were used for quantification of relative change in gene expression, by the 2^−ΔΔCt method (Livak and Schmittgen, 2001).

2.6. Analysis of secondary metabolites

2.6.1. Detection of indole-3-acetic acid (IAA)

For induction of IAA biosynthesis, the cultures were raised in SSM + 1 mM Trp for 20 h and subsequently subcultured in SSM with 5 mM Trp. The amount of IAA was quantified by HPLC (Malhotra and Srivastava, 2006). Briefly, the culture filtrate samples extracted with ethyl acetate were vacuum dried (Eyela, Japan) and reconstituted in 2 ml of methanol of which 10 μl aliquots were resolved on Merck Lichrospher 100 RP-18e (250 mm × 4 mm, 5 μm; Darmstadt, Germany) column in a class10 Shimadzu HPLC system (Class VP release 6.13 SP1; Kyoto Japan). Samples and standards (Sigm-Aldrich St. Louis Mo. USA) were analyzed in methanol: 1% acetic acid (40:60 v/v) at a flow rate of 1 ml/min at 280 nm with a UV detector. Quantification was done by the peak area in comparison to the standard as described earlier (Malhotra and Srivastava, 2006). The IAA amount was represented as μg IAA/OD600 of grown cells so as to normalize it against any variation in the growth.

2.6.2. Detection of hydrogen cyanide (HCN) production

Qualitative determination of the production of HCN was performed by the alkaline picate test as described by earlier (Lorck, 1948).

2.6.3. Estimation of siderophore and phenazines production

The detection of siderophores was performed by chrome Azurol S (CAS) method (Schwyn and Neilands, 1987). Spectral analysis of culture supernatants of different strains grown in SSM, in wavelength range of 100–800 nm to detect maximum absorption of siderophores was carried out. Phenazine was extracted and quantified according to the protocol described earlier (Upadhyay and Srivastava, 2008; Whistler and Pierson, 2003).

2.7. Evaluation of biocontrol potential

2.7.1. Anti-fungal potential

Plant growth-promoting functions of these strains were also evaluated in terms of their biocontrol properties. Antagonistic ability of strain Psd and all the three recombinant strains was initially checked in vitro against two wilt causing plant fungal pathogens, Fusarium oxysporum and Verticillium dahliae. Antibiosis by the test strain against the fungal pathogens was assessed on the basis of inhibition zone after 48 h of incubation at 25 °C. For this the bacterial cultures were raised in LB medium in the presence of appropriate antibiotics for plasmid selection. The bacterial culture supernatant was concentrated using Speed-vac (Eyela, Japan) and

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Table 2

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer Sequence</th>
<th>Site</th>
<th>Reverse Primer Sequence</th>
<th>Site</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
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<tr>
<td>rsmX</td>
<td>ATAGGAACCAACAAGACGGCGAGAA</td>
<td>BamHI</td>
<td>TAAACGTTTTTTTGAGACCATGACCTC</td>
<td>HindIII</td>
<td>100</td>
</tr>
<tr>
<td>rsmY</td>
<td>ATAGGAACCAACAAGACGGCGAGAA</td>
<td>EcoRI</td>
<td>ATTGACCGGTTGGCTGAGACCTC</td>
<td>BamHI</td>
<td>114</td>
</tr>
<tr>
<td>rsmZ</td>
<td>ATAGGAACCAACAAGACGGCGAGAA</td>
<td>KpnI</td>
<td>TTAGCGATACGAAACGCAACC</td>
<td>Xhol</td>
<td>100</td>
</tr>
<tr>
<td>gacA</td>
<td>ATAGGAACCAACAAGACGGCGAGAA</td>
<td>XhoI</td>
<td>TAGCGACTAGCAGTCTCTCAGC</td>
<td>SalI</td>
<td>181</td>
</tr>
<tr>
<td>gen</td>
<td>ATAGGAACCAACAAGACGGCGAGAA</td>
<td>ZruI</td>
<td>ATAGAGCTCTTAGGCGAGTACCTGG</td>
<td>ZruI</td>
<td>538</td>
</tr>
</tbody>
</table>

16 s rDNA AAGCAAAGCAAGAACTTA – CACCCGCTCTCTTGAG 200

Note: Nucleotides in bold represent restriction sites.
100 µl of the same was put in the wells cut out in fungus- inoculated PDA plates.

2.7.2. Spore germination test

_Pseudomonas_ strain Psd harboring pME7320, pME6918 and pME6359 were raised in the presence of appropriate antibiotics to maintain plasmid selection. The wild-type strain Psd was raised in the absence of antibiotics. The bacterial culture supernatant from modified strains along with wild-type strain Psd, was diluted to 50% in Peptone Dextrose (PD) broth and was used to test the effect on spore germination of _F. oxysporum_ and _V. dahliae_ after overnight incubation. Germination was assessed by microscopic observation (Olympus BX51). The control set was raised in PD medium similarly diluted with SSM.

2.8. Plant assays

_Tomato_ (Solanum lycopersicum var. Pusa Ruby) was used to monitor the anti-fungal action of the secondary metabolites produced by strain Psd and its variants. Overnight water- soaked tomato seeds were sown in vermiculite placed in an egg tray and kept for 20–30 days for growth at 28 °C and watered regularly until plants reached the 4-leaf-stage. Such seedlings were then planted in the 30 ml culture tubes containing ~15 g of autoclaved garden soil mixed with three parts of maize flour. The test set, each containing 10 seedlings in triplicates contained plants that were inoculated with ~10⁸ spores of _F. oxysporum_ and _V. dahliae_ suspended in culture extract of Psd and all the three recombinant strains. Another set contained fungal spores (~10⁹/ml) suspended in normal saline (0.85% NaCl). The control tubes contained plants without any exposure to fungal spores. Plant were watered regularly with nutrient solution and maintained at 28 °C with 16 h: 8 h (light: dark) period. Growth of the plants was monitored for 15 days.

2.9. Statistical analysis

All bacterial culture experiments were carried out in three independent sets, each consisting of 3 replicates. Values shown here represent mean ± standard error of the mean (SEM). The plant experiments were performed in triplicates with 10 seedlings per set. Statistical analysis was performed using Dunnet’s t-test.

3. Results

3.1. PCR amplification and sequence analysis of small regulatory RNA genes

PCR amplification of _rsmY_ (102 bp) and _rsmZ_ (114 bp) genes from strains Psd, Psd-30 and the control strain _P. protegens_ CHAO is shown in Fig. 1A and B. However, _rsmX_ could not be amplified in the strains Psd and Psd30. The amplified fragments were gel eluted, purified, and digested with appropriate restriction enzymes. These were then directionally cloned in pBKCS(+) and sequenced. The retrieved sequences confirmed the presence of _rsmY_ and _rsmZ_ in strain Psd, indicating the presence of sRNAs in the Psd genome. The sequences revealed the presence of the six characteristic 5'-GGA-3' motifs in both _rsmY_ and _rsmZ_ (Fig. 2A). Sequences for both the genes were deposited in NCBI database as accession numbers FJ888393 (_rsmY_) and EU491520 (_rsmZ_). The retrieved _rsmY_ and _rsmZ_ sequences from strain Psd revealed high similarity with _P. aeruginosa_ strain PAO1 and _P. protegens_ strain CHAO.

3.2. Generation of recombinant strains

The plasmids, pME7320, pME6359 and pME6918, were originally obtained from _P. protegens_ CHAO. To ease the selection process in strain Psd, these plasmids were modified by incorporating _kan_ or _gen_ gene and the recombinant plasmids (~10.9 kb) were labeled as pME7320-kan, pME6359-kan and pME6918-gen. Following electro-transformation in strain Psd and selection on respective antibiotics, the transformants were also confirmed by plasmid isolation and restriction digestion. Modified strains expressing CHAO _rsmX_, _rsmY_, and _rsmZ_ were labeled as Psd-pME7320, Psd-pME6918, and Psd-pME6359, respectively.

3.3. Expression analysis

Expression analysis was performed for the over-expression constructs of _RsmY_ and _RsmZ_ using qPCR. Approximately 4-folds up-regulation in the level of _rsmY_ was obtained in strain Psd-pME6918 (Fig. 2B). Similarly, a 3-folds up-regulation was obtained in Psd-pME6359 for _rsmZ_. Additionally, expression analysis was also carried out for the genes encoding the response regulator _gacA_ and one of the RNA-binding proteins, _rsmA_, in the modified strains. Although the over-expression of _rsm_ genes in strain Psd resulted in significant upregulation of _gacA_ transcript in all the modified strains, the maximum (approx. 3-folds) upregulation was observed in strain Psd-pME7320. Besides, a significant down-regulation in the transcript levels of _rsmA_ were observed in all the strains (Fig. 2B). The maximum down-regulation (approx. 4-folds) was observed in _rsmY_ overexpressing strain, Psd-pME6918.

3.4. Biocontrol potential

The ability of the _rsm_ over-expressing strains to inhibit phytopathogenic fungi was evaluated against _F. oxysporum_ and _V. dahliae_ on the basis of their respective inhibition zones. The variant strains (Psd-pME7320, Psd-pME6918 and Psd-pME6359) showed almost double the size of zone of growth inhibition as compared to the wild type strain Psd (Figs. 3A; S1 in the online version, at http://dx.doi.org/10.1016/j.micres.2016.12.006). In order to determine the basis of the antifungal potential, spore germination test was also conducted. Normal germination of spores followed by mycelial formation was observed in the control sets, wherein _F. oxysporum_ and _V. dahliae_ spores were suspended in PD: SSM (1:1) medium. Addition of culture filtrates from wild-type strain Psd and the Psd variants, however, displayed a drastic inhibition in the fun-
**Fig. 2.** A) Organization of rsmY and rsmZ gene sequences in strain Psd. GGA elements are indicated in gray boxes B) qPCR for assessment of relative expression of rsmY and rsmZ and rsmA in the rsm overexpressing strains (*P < 0.05; **P < 0.01; ****P < 0.0001; ns not significant).

3.5. Secondary metabolite production

The over-expressing strains were further characterized for the production of different antimicrobials and antibiotics produced by the wild type strain Psd.

3.5.1. Total phenazine

Since these genes in the recombinant strains are placed under P_tac, phenazine production was also estimated by inducing the cells with 1 mM IPTG. As is clear from Fig. 4A, the modified strains produced enhanced levels (∼3-folds) of phenazine in comparison to wild type cells. Maximum induction was observed in the cells over-expressing rsmY, Psd-pME6918.

3.5.2. Indole acetic acid

Higher IAA production was obtained in all the modified strains when compared with strain Psd (Fig. 4B). The increase was maximum in case of Psd-pME6918, indicating towards the role of rsmY in IAA biosynthesis by the strain.

3.5.3. HCN

The recombinant strains produced differential amounts of HCN compared to the parent strain Psd. Among the three variants, rsmY over-expressing strain Psd-pME6918 was found to show slightly higher amount of HCN as detected qualitatively (Fig. S3 in the online version, at http://dx.doi.org/10.1016/j.micres.2016.12.006).

3.5.4. Siderophores

Spectral analysis of culture supernatant of strains grown in SSM showed similar levels of siderophore production by all the strains (Fig. S2 in the online version, at http://dx.doi.org/10.1016/j.micres.2016.12.006).

3.6. Plant assays

Assessment of effect of overexpression of rsm genes on biocontrol properties revealed a higher potential of the modified strains (Psd-pME7320, Psd-pME6918, and Psd-pME6359) in biocontrol of plant fungal pathogens. This was further tested by co-inoculation of culture filtrates from these strains with spores of phytopathogenic fungi *F. oxysporum* and *V. dahliae* and monitoring the disease development in tomato plants. As can be seen in Figs. 5 and S4 in the online version, at http://dx.doi.org/10.1016/j.micres.2016.12.
has also been earlier documented in the strain, pointing towards the presence of a regulatory cascade involved in these processes (Upadhyay and Srivastava, 2008). Phosphorylation of GacA positively controls the transcription of small regulatory RNAs, like RsmX, RsmY and RsmZ in the biocontrol strains (Haas and Defago, 2005; Wang et al., 2013; Harfouche et al., 2015; Li et al., 2015). The present investigation has identified the presence of two sRNAs, RsmY and RsmZ, in strain Psd. These sRNAs seem to control expression of other genes or operons at the post-transcriptional level, commonly upon stress or auto-induction signaling.

While the presence of rsmY and rsmZ genes was established in the strain Psd, rsmX could not be detected in the strain during the presence study. This was in line with the observation that several bacterial species, such as P. aeruginosa and E. coli, possess only two sRNAs (Miller et al., 2016). While multiple homologous copies of rsmX are found in Pseudomonads like P. syringae, P. protegens strain Pf-5 and P. fluorescens strain Pf0-1, some other Pseudomonads are reported to lack rsmX (Gonzalez et al., 2008). In P. aeruginosa, the GacS-GacA pathway controls only two sRNAs, RsmY and RsmZ, which are functionally redundant (Brencic et al., 2009; Frangipani et al., 2014). Mutation in the sRNA genes are known to have similar phenotypic effects like GacS/GacA mutations (Wang et al., 2013; Song et al., 2015). Rsm sRNAs contain several stem-loops with an invariant GGA trinucleotide exposed in the hairpin loop serving as the characteristic structural sequence motifs relevant for sRNA activity and stability (Valverde, 2009; Harfouche et al., 2015; Miller et al., 2016). Analysis of the retrieved sequences of rsmY and rsmZ genes revealed the presence of characteristic GGA motifs, which shared strong sequence similarity with other pseudomonads.

A number of studies have highlighted the role of GacA in controlling cellular processes, such as biofilm formation, motility and secretion of biocontrol factors, including HCN, phenazine, 2,4 DAPG, pyoluteorin, proteases and siderophores (Lalaouna et al., 2012; Wang et al., 2013; Li et al., 2015). A gacA mutation in P. chlororaphis impaired the production of HCN, phenazine and extracellular proteases (Li et al., 2015). Similarly, gacA mutation abolished the production of lipases in P. protegens Pf-5 (Zha et al., 2014). On the other hand, overexpression of the sRNAs suppresses the effect of gacA mutation, which has been shown in several Pseudomonads (Valverde et al., 2003; Gonzalez et al., 2008; Lalaouna et al., 2012). In the present study, the functional dependence of GacA on sRNAs was revealed by an upregulation of gacA in the rsm overexpressing variants of strain Psd. Interestingly, maximum upregulation was obtained in rsmX overexpressing strain Psd-pME7320, harboring rsmX from P. protegens strain CHA0 (Fig. 6). Since the wild-type strain Psd lacks rsmX, this observation hints to the cross-species conservation of the sRNAs in Pseudomonas.

Small regulatory RNAs influence the translation of target mRNAs by sequestering RNA-binding regulatory proteins, such as CstA in E. coli and RsmA in Pseudomonas sp (Harfouche et al., 2015). Multiple homologs of RsmA have been reported in Pseudomonas sp, including RsmE of P. protegens CHA0 and RsmN of P. aeruginosa (Kay et al., 2005; Morris et al., 2013). The role of sRNAs in relieving the translational repression exerted by RsmA and RsmE has been demonstrated earlier for a number of Pseudomonads, including P. protegens strains Pf-5 and CHA0 and P. chlororaphis strain 30–84 (Brencic et al., 2009; Valverde, 2009; Workentine et al., 2009; Hassan et al., 2010; Wang et al., 2013). Moreover, the overexpression of small regulatory RNAs has been demonstrated to titrate more RsmA and RsmE, thereby overcoming the translational repression of target genes (Valverde et al., 2004; Valverde, 2009).

In light of this, the transcriptional profiling of rsmA gene in the rsm

![Fig. 3. A) Agar well diffusion assay for antifungal activity against F. oxysporum. The results of these assays depicted the enhanced antifungal property of overexpressing strain B) Effect of 50% diluted culture filtrates of Psd, Psd-pME7320, Psd-pME6918 and Psd-pME6359 on the spore germination of F. oxysporum (right panel).](image-url)
overexpressing constructs of strain Psd was performed. Although rsmA transcript levels were significantly low in all the modified strains, maximum reduction of about 75% was obtained in the rsmY overexpressing strain, Psd-pME6918 (Fig. 6). Similar results have been reported in P. protegens and P. aeruginosa where secondary metabolite production was regulated by RsmY and RsmZ by sequestering RsmA (Kay et al., 2005; Gottesman and Storz, 2011). Moreover, RsmA was demonstrated to have a higher affinity for RsmY, when present in lower concentrations (Valverde et al., 2003). Also, an rsmA mutation led to reduced rsmY expression, indicating towards a strong correlation between the two genes (Valverde et al., 2003).

GacS-GacA, in conjunction with Rsm sRNAs, has been known to regulate a number of cellular functions. Strain Psd has been shown to produce a number of antifungal metabolites, including broad-host range antibiotics, HCN, and siderophores, and the same is reflected in the significant inhibition of F. oxysporum and V. dahliae in both in vitro and in planta experiments (Upadhyay and Srivastava, 2008, 2010a,b). The over-expression of rsm genes in the strain brought about some remarkable changes in terms of increased expression of the response regulator, GacA and down-regulation of the RNA-binding protein, RsmA. This translated into the altered biocontrol potential of the variant strains. A significant enhancement in total phenazine and HCN production indicated towards the role of the sRNAs in regulation of these processes. Besides, higher levels of IAA production were also recorded in the recombinant strains. This could be explained on the basis of IAA production by tryptophan side chain oxidase (TSO) pathway which is also reported to be regulated by sRNAs (Workentine et al., 2009). The culture filtrates from the rsmX, rsmY and rsmZ overexpressing strains resulted in remarkable decrease in spore germination and, eventually, mycelium formation by target fungi, F. oxysporum and V. dahliae. The biocontrol property was also reflected in the efficient bio-protection of tomato seedlings from F. oxysporum and V. dahliae infections by the variant strains, as compared to wild-type Psd. This supports the earlier proposition of the involvement of sRNAs in regulation of biocontrol traits in strain Psd. The application of the variant strains, however, has to be further validated under field conditions.

5. Conclusions

The present study highlights the role of sRNAs in regulation of biocontrol traits in fluorescent Pseudomonas strain Psd. The functionality of rsm genes belonging to the Gac/Rsm signal transduction pathway in the regulation of phenazines and other genes important
for biocontrol in strain Psd was examined. Additionally, the study also indicated a direct role of sRNAs in regulation of the expression of GacA as well as the RNA-binding protein, RsmA, thus highlighting the crucial importance of the sRNAs in maintaining bacterial physiology.

**Conflict of interest**

Authors declare no conflict of interests.

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**References**

One page of a scientific document with a focus on the regulation of gene expression and biocontrol agents. The text includes references to key studies and concepts such as GacA, Rsm, and regulatory pathways in bacteria. The document discusses the role of RNA-binding proteins in the regulation of gene expression and their impact on biocontrol and antibiotic production.