A Regulatory RNA (PrrB RNA) Modulates Expression of Secondary Metabolite Genes in Pseudomonas fluorescens F113

SIMON AARONS,† ABDELHAMID ABBAS, CLAIRE ADAMS, ANNE FENTON,‡ AND FERGAL O’GARA*

BIOMERIT Research Centre, Department of Microbiology, National University of Ireland, Cork, Cork, Ireland

Received 10 January 2000/Accepted 24 April 2000

The GacS-GacA two-component signal transduction system, which is highly conserved in gram-negative bacteria, is required for the production of exoenzymes and secondary metabolites in Pseudomonas spp. Screening of a Pseudomonas fluorescens F113 gene bank led to the isolation of a previously undefined locus which could restore secondary metabolite production to both gacS and gacA mutants of F113. Sequence analysis of this locus demonstrated that it did not contain any obvious Pseudomonas protein-coding open reading frames or homologues within available databases. Northern analysis indicated that the locus encodes an RNA (PrrB RNA) which is able to phenotypically complement gacS and gacA mutants and is itself regulated by the GacS-GacA two-component signal transduction system. Primer extension analysis of the 132-base transcript identified the transcription start site located downstream of a ω9 promoter sequence from positions −10 to −35. Inactivation of the prrB gene in F113 resulted in a significant reduction in 2,4-diacetylphloroglucinol (Phl) and hydrogen cyanide (HCN) production, while increased metabolite production was observed when prrB was overexpressed. The prrB gene sequence contains a number of imperfect repeats of the consensus sequence 5’-AGGA-3’, and sequence analysis predicted a complex secondary structure featuring multiple putative stem-loops with the consensus sequences predominantly positioned at the single-stranded regions at the ends of the stem-loops. This structure is similar to the CsrB and RsmB regulatory RNAs in Escherichia coli and Erwinia carotovora, respectively. Results suggest that a regulatory RNA molecule is involved in GacA-GacS-mediated regulation of Phl and HCN production in P. fluorescens F113.

Pseudomonas fluorescens F113 was isolated as a biocontrol agent for the control of Pythium ultimum-mediated damping-off of sugar beet (35). Inhibition of Pythium ultimum has been attributed to the production of the antimicrobial agent 2,4-diacylphloroglucinol (Phl) (11). However, the strain also synthesizes hydrogen cyanide (HCN) and an exoprotease. These secondary metabolites and exoprotease have previously been shown to be positively regulated by the GacS (previously LemA) and GacA two-component signal transduction system (8) common to numerous Pseudomonas spp., including P. syringae (31), P. viridiflava (18), P. aeruginosa (30), and P. fluorescens (6, 13, 17, 32). Sensor proteins such as GacS are typically transmembrane proteins that respond to environmental stimuli by autophosphorylation, followed by transfer of the phosphate to the cognate response regulator, in this case GacA. The GacA response regulator contains a DNA binding motif and is thought to activate or repress genes directly by binding to the target gene promoter. However, direct binding of GacA to putative target promoters has yet to be demonstrated.

Recent research in P. aeruginosa PAO (30) has revealed that the GacS-GacA signal transduction system contributes to a larger regulatory cascade involving acyl-homoserine lactone-mediated quorum sensing and alternate sigma factors. Indeed, Reimann et al. (30) demonstrated that GacA positively controls the production of N-butylhomoserine lactone. Furthermore, N-butylhomoserine lactone was demonstrated to regulate virulence factors, such as pyocyanin, cyanide, and lipase, and to activate the transcription of rpoS, which encodes the post-exponential phase and stress response sigma factor σ8. The potential for additional factors to be involved in GacS regulation was demonstrated by Kitten and Willis (15). This research revealed that overexpression of the ribosomal proteins L35 and L20 could partially complement the gacS (lemA) mutant phenotype of P. syringae.

P. fluorescens F113 gacS mutants and gacA mutants do not synthesize Phl, HCN, or exoprotease (8). These phenotypes are restored upon complementation in trans with the respective genes. However, the direct activation of the genes responsible for these phenotypes through GacA binding has yet to be demonstrated. Indeed, activation of secondary metabolites and exoenzymes in P. fluorescens F113 may involve a more complex regulatory cascade, and evidence for this is presented here with the description of the prrB gene encoding a regulatory RNA molecule.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. P. fluorescens F113 and derivatives were routinely grown at 28°C in sucrose asparagine medium (34). The medium was supplemented, where indicated, with 100 μM FeCl3 for high-iron conditions. Escherichia coli strains were grown at 37°C in Luria-Bertani (LB) broth or agar. Antibiotics when required, were added to the medium at the following concentrations: for tetracycline, 25 μg ml−1 for E. coli and 75 μg ml−1 for P. fluorescens; for chloramphenicol, 30 μg ml−1 for E. coli and 200 μg ml−1 for P. fluorescens; and for kanamycin, 25 μg ml−1 for E. coli and 50 μg ml−1 for P. fluorescens.

Construction of pCU300 derivatives. The P. fluorescens F113 genomic DNA fragment was subcloned from pCU300 as a BamHI-HindIII fragment into the broad-host-range (BHR) vector pBBR1MCS to form pCU301. Sau3A-BamHI fragments from pCU300 were subcloned into pBBR1MCS to form pCU302 and pCU303. The M13/pUC reverse primer (P1) (5’-AGGGATAAACAATTTCAC-3’) and primer P2 (5’-CTGATATCCCTTGCTTCCG-3’), which incorporate a EcoRV site, were used to amplify the loci that was cloned in...
pHP45-Tc (27) and blunt end ligated into the pK18 (28). The resulting double-crossover transformants were selected as Tcr and Km resistant. The resulting plasmid pCU305 was subcloned into the Bam HI sites of pBBR1MCS to form pCU305.

Sodium citrate (0.05 M) buffer. Probe labeling was carried out by end labeling primers P3 and P4 using 9 32P]ATP followed by PCR amplification using pCU305 as the template. The resulting 228-bp PCR product containing the prrB gene was purified using a High-purity-PCR purification kit (Boehringer). Hybridization was performed at 65°C overnight in 0.25 M NaH2PO4–7% sodium dodecyl sulfate and washed blots were examined by autoradiography with X-ray film (BIOMAX, Kodak). To measure the size of the PrR transcript, a 145-bp DNA fragment was PCR amplified using primers P4 and prrBT7 (5'-TTCGAGTCCGAGAAA TCGGACGGCG-3'), which carries a Bam HI site, and amplified from pCU301; Cmr This study

Bacterial strains

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<th>Bacterial strain or plasmid</th>
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* Ap⁺, ampicillin resistant; Cm⁻, chloramphenicol resistant; Km⁺, kanamycin resistant; Tc⁻, tetracycline resistant.

pBR1MCS to form pCU304. A 228-bp fragment containing the putative prrB gene was amplified by PCR using the primers P3 (5'-CGTACGGGATCCGGACGCA-3'), which carries a Kpn I site, and P4 (5'-TTCGAGTCCGAGAAA TCGGACGGCG-3'), which carries a Bam HI site, and cloned into the KpnI-BamHI sites of pBR1MCS to form pCU305.

**Construction of F113prrB mutant.** The BamHI-XhoI fragment of pCU300 was subcloned into the BamHI-SalI sites of the narrow-host-range (NHR) vector pK18 (28). The Ω-Tc fragment was isolated as a Small fragment from plasmid pHP45-Tc (27) and blunt end ligated into the SalI site (33) within prrB. The resulting pCU306 suicide construct was introduced into F113 by electroporation, and double-crossover transformants were selected as Tc⁻ and Km⁻. The resulting prrB mutant (FRB1) in which the Ω-Tc fragment had inserted within the chromosomal prrB copy was verified by Southern and Northern blot hybridization.

**Exoproduct assays.** Phl synthesis was assayed qualitatively using the *Bacillus* inhibition plate bioassay described previously (11). Pseudomonas test strains were assayed for Phl production by high-performance liquid chromatography as previously described (35). Proteolytic activity was assayed qualitatively using skim milk agar plates (9). Briefly, strains were streaked onto the plates and incubated for 72 h at 30°C, and then the diameters of the clearing zones were compared. Hydrogen cyanide production was detected qualitatively using the filter paper assay described previously (3). Quantification of hydrogen cyanide was performed as described previously (38).

**DNA manipulations and cloning procedures.** Small- and large-scale plasmid DNA isolation was performed using Qiagen Plasmid Mini and Maxi kits, respectively, according to the manufacturer’s specifications (Qiagen). Restriction digestion and ligation procedures were performed by the methods of Sambrook et al. (33). Chromosomal DNA was isolated using the method of Chen and Kuo (5). DNA manipulations and cloning procedures. Small- and large-scale plasmid DNA isolation was performed using Qiagen Plasmid Mini and Maxi kits, respectively, according to the manufacturer’s specifications (Qiagen). Restriction digestion and ligation procedures were performed by the methods of Sambrook et al. (33). Chromosomal DNA was isolated using the method of Chen and Kuo (5).

**RESULTS**

Identification of a locus that restores Phl, HCN, and exoproduct production to gacS and gacA mutants. To isolate genes capable of complementing the *P. fluorescens* F113 gacS and gacA mutants, a BamHI plasmid library cloned in the BHR plasmid pSUP106 (36) was mobilized into the F113gacS mutant strain FL33 (8) and screened for restoration of Phl production using the standard *Bacillus* bioassay (11). Plasmids which restored the Phl-synthesizing ability to FL33 were introduced into the F113 gacA mutant, FG9, and transconjugants in both strains were further characterized for protease and HCN production using the standard bioassays (see references 9 and 10).

**Nucleotide sequence determination and sequence analysis.** The nucleotide sequence of the prrB region was determined by primer walking using an Applied Biosystems PRISM 310 Automated Genetic Analyser (Perkin Elmer). The sequence data were assembled using DNASTAR software package (DNASTAR, Madison, Wis.) and analyzed using the University of Wisconsin Genetic Computer Group (GCG) program FASTA (26) and BLAST (1) at the National Center for Biotechnology Information (Bethesda, Md.).
and transconjugants did not produce Phl, HCN, or exoprotease. The pMP220 derivative was conjugated into strains FL33 and FG9, respectively. A single plasmid, pCU300, was identified which restored Phl, HCN, and protease production to both mutant strains FL33 and FG9.

To further define the region responsible for multicopy suppression of the mutant phenotypes, restriction fragments from the pCU300 insert were subcloned in the BHR vector pBBR1MCS (16), and derivatives were then screened to identify the smallest cloned fragment which could complement both the FL33 and FG9 mutant phenotypes. A 2.8-kb subclone of pCU300 in pBBR1MCS, designated pCU301, complemented the mutant phenotypes, pCU301 contained an essential SalI site in that two BamHI-SalI subclones of pCU301 (pCU302 and pCU303) did not complement FL33 and FG9. In order to determine a more precise location of the region required for phenotypic complementation of the mutants, a 850-bp region of pCU301 was PCR amplified using the primers P1 and P2. The PCR product was cloned into pBHR1MCS as a HindIII-EcoRV fragment to form pCU304, and this plasmid was found to restore Phl and HCN production to both FL33 and FG9 mutants (Fig. 1) and also significantly increased levels of Phl and HCN in the wild-type F113 when introduced in trans.

The HindIII-EcoRV fragment from pCU304 was also subcloned into the multiple cloning site of the promoter-probe vector pMP220 (37). This vector was originally constructed so as to have no exogenous promoter activating transcription through the multiple cloning site and also exists in P. fluorescens in low copy (approximately one to five copies) (37). The pMP220 derivative was conjugated into strains FL33 and FG9, and transconjugants did not produce Phl, HCN, or exoprotease when assessed using standard bioassays (data not shown). This shows that prrB, under the control of its own promoter, does not complement the mutant phenotype of FL33 and FG9 and strongly suggests that prrB expression is under GacA-GacS control.

Nucleotide sequence and characterization of the complementing locus. The 850-bp HindIII-EcoRV subclone was completely sequenced in both directions using universal primers and a primer walking strategy. Comparison with nonredundant nucleotide and protein databases using FASTA (26) and BLAST (1) protocols did not show any obvious homologues. Furthermore, none of the putative open reading frames determined using DNASTAR software had identifiable ribosome binding sites or exhibited typical Pseudomonas codon usage (24).

Phenotypic complementation analysis with pCU301, pCU302, and pCU303 subclones, however, revealed that the region immediately surrounding the SalI site was essential for complementing F113 gacS and gacA mutant phenotypes. Sequence analysis of this region revealed a candidate gene that spanned the SalI site and contained putative −10 and −35 sites and a Rho-independent terminator sequence. Located within the sequence were numerous imperfect repeats of the consensus sequence 5′-AGGA-3′ (Fig. 2A). The predicted RNA was surveyed using mfold (39). Results of this analysis revealed a complex secondary structure featuring multiple putative stem-loop structures. The 5′-AGGA-3′ consensus sequences were positioned at the end of predicted hairpin loops distributed throughout the molecule and in single-stranded segments between the loops (Fig. 2B). Excluding the apparent Rho-independent terminator, four of five hairpin loop structures contained the consensus sequence. This structure resembles that of the carbon storage regulatory RNA (CsrB) of E. coli (20) and the regulatory RNA (RsmB) of Erwinia carotovora (22). To determine whether this putative gene was sufficient for the suppression of the F113 gacS and gacA mutations in trans, the candidate gene was PCR amplified using primers P3 and P4 (Fig. 2A) and cloned into pBHR1MCS such that the −35 region was immediately downstream of the plasmid-encoded Phe promoter. The resulting construct pCU305 was conjugated into FL33 and FG9 and found to restore Phl, exoprotease, and HCN production using standard bioassays (data not shown).

Northern analysis revealed a small RNA molecule that is regulated by GacS-GacA. To determine whether pCU305 encoded an RNA transcript, Northern analysis was conducted with P. fluorescens F113 and the mutant strains FL33 and FG9 in the presence and absence of pCU305. Total cellular RNA was isolated from late-log-phase cultures of F113, F113/pBBR1MCS, FL33, FL33/pCU305, FG9, and FG9/pCU305 and was probed with the radiolabeled 228-bp fragment amplified from pCU305 using P3 and P4 primers. Northern blot hybridization with this probe detected a single major transcript of approximately 130 nucleotides which was present in F113, F113/pBBR1MCS, FL33/pCU305, FL33/pCU305, FG9, and FG9/pCU305 and was probed with the radiolabeled 228-bp fragment amplified from pCU305 using P3 and P4 primers. Northern blot hybridization with this probe detected a single major transcript of approximately 130 nucleotides which was present in F113, F113/pBBR1MCS, FL33/pCU305, FL33/pCU305, FG9, and FG9/pCU305 and was probed with the radiolabeled 228-bp fragment amplified from pCU305 using P3 and P4 primers.

The putative Pseudomonas regulatory RNA molecule was designated PrrB RNA. Increased prrB transcript levels were observed in the wild type F113 and F113prrB mutant in the presence of pCU305 compared with FL33/pCU305 and FG9/pCU305. It was also interesting to note that FG9/pCU305 produced less prrB transcript than FL33/pCU305.

Determination of the transcription start site of PrrB RNA and identification of potential promoter elements. To identify the promoter responsible for prrB transcription, total RNA isolated from wild-type P. fluorescens F113 grown in minimal

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**FIG. 1.** Effect of pCU304 on Phl (A) and HCN (B) production by wild-type F113 and F113pCU304 (FL33) and F113pCU304 (FG9) mutants. Introduction of the plasmid pCU304 in trans into both FL33 and FG9 mutants restored Phl and HCN production and increased Phl and HCN production in the wild type.
FIG. 2. (A) Nucleotide sequence of prrB. The first line of the sequence shows the transcription start (str) and promoter organization; −10 and −35 sequences are boxed. Putative KDGR recognition sites are boxed. The potential hairpin loop structures are identified by arrows and the 5′-AGGA-3′ imperfect repeat motifs are in boldface type. The nucleotide sequence of primers P3 and P4 used for the construction of pCU305 are underlined. (B) Secondary structure prediction for PrrB RNA generated using mfold. Note that all the repeated elements are predicted to reside in the single-stranded regions and five of the nine are specifically found in the hairpin stem-loop. (C) Nucleotide sequence alignment of Erwinia KDGR boxes with putative prrB KDGR sequences.
medium with sucrose as the carbon source was subjected to primer extension analysis (29). This analysis revealed only one specific transcript starting with the 5′ sequence TGT and identifying the transcriptional start site 9 bases downstream of the putative −10 TAATAT promoter sequence (Fig. 4).

Further analysis of the sequence upstream of the transcription start site revealed inverted repeat sequences surrounding the −35 site (Fig. 2A). It is noteworthy that the inverted repeat sequences lie in close proximity to the RNA polymerase recognition sequence of the promoter.

Sequences upstream of the rsmB gene of E. carotovora contain three binding sites recognized by the regulatory protein KdgR_{Ecc} (23). KdgR_{Ecc} negatively regulates rsmB at the transcriptional level. Sequence analysis of the coding region of prrB revealed a sequence homologous to the KdgR_{Ecc} consensus sequences (Fig. 2A and C). Furthermore, a sequence between positions −77 and −93 from the transcription start site of prrB is highly similar to the known consensus sequence recognized by KdgR_{EcoB}. KdgR_{EcoB} is a repressor that negatively regulates expression of many genes involved in pectinolysis and pectinase secretion in Erwinia chrysanthemi (25).

Construction of a PrrB mutant of P. fluorescens F113. In order to disrupt prrB, a Smal Ω-Tc fragment from pHP45Ω-Tc (27) was blunt end ligated within the internal SalI site in the BamHI-XhoI fragment from pCU300 and cloned in the NHR vector pK18 (28). This suicide construct, pCU306, was electroporated into P. fluorescens F113, and double-crossover recombinants were selected as being Tc R and Km’. The presence of the Ω-Tc insertion within the prrB gene of the mutant F113prrB was confirmed by Southern hybridization analysis (data not shown). A PrrB-negative phenotype was demonstrated by Northern hybridization analysis when total cellular RNA was isolated from late-log-phase cells and probed with the 228-bp fragment of pCU305 (Fig. 3). The PrrB transcript was restored in the prrB mutant when pCU305 was introduced in trans.

In qualitative plate assays, the prrB mutant FRB1 did not display obvious changes in Phl, HCN, or exoprotease production from that of the wild type (data not shown). However, quantitative assays showed that there was a significant decrease in Phl production by the F113prrB mutant during the mid- to late log phase of growth (Fig. 5).

**DISCUSSION**

A novel gene, prrB, has been identified in the biocontrol strain P. fluorescens F113. The prrB gene was found to restore the production of Phl, HCN, and protease to gacS and gacA mutants of P. fluorescens F113. From sequence analysis, prrB is not predicted to encode a protein but was demonstrated to synthesize a small RNA molecule (PrrB RNA) which may act as a regulatory RNA molecule.

Northern analysis suggested that prrB is regulated, directly or indirectly, through the GacS-GacA two-component signal transduction system (Fig. 3), as the PrrB transcript was not
detectable in either the gacA or gacS mutant. Also, when cloned in the promoterless vector, prrB did not restore secondary metabolite production in either the gacS or gacA mutant. The lower levels of prrB transcript produced by FL33/pCU305 and FG9/pCU305 compared with that of the wild-type F113 and F113*prrB mutant in the presence of pCU305 may also reflect a role for GacA and GacS in the regulation of prrB.

It was interesting to note that FG9/pCU305 produced less prrB transcript than FL33/pCU305. The reason for this is unclear but could suggest uncoupling of GacA and GacS regulation in relation to prrB. To date, the mechanism of regulation by the GacA-GacS two-component system has not been completely elucidated, and although GacA has a putative DNA binding helix, the target promoters recognized by activated GacA have yet to be demonstrated. Furthermore, analysis of secondary metabolite regulation in P. aeruginosa (30) predicts that this two-component system may activate target genes through a complex regulatory cascade. Phenotypic complementation of gacS and gacA mutants by PrrB RNA suggests that PrrB may function as a regulator within a P. fluorescens GacS-GacA regulatory cascade. However, although inactivation of prrB reduces Phl and HCN production, this did not prevent synthesis of Phl, HCN, or exoprotease. Thus, PrrB RNA does influence secondary metabolite synthesis but not strongly and could be in response to some extra- or intracellular signal or as a stress response. It was interesting that the F113*prrB mutant exhibited delayed Phl production, predicting that PrrB RNA may be involved in the early induction of certain secondary metabolite biosynthesis. The prrB dosage experiments mimic, to a degree, results obtained for P. aeruginosa GacA gene dosage experiments (30). In P. aeruginosa, inactivation of gacA resulted in temporal delay (an optical density at 600 nm [OD600] of 1.2) and reduction of cyanide production. Conversely, when gacA is overexpressed, cyanide production starts much earlier at an OD600 of 0.1. Similarly, in F113, inactivation of prrB delays Phl production (Fig. 5), and in the presence of more copies of prrB, Phl production is induced to maximum levels at low cell density (8).

Recently, Blumer et al. (2) demonstrated that in P. fluorescens CHAO, the GacA-GacS two-component system can mediate posttranscriptional regulation possibly via a recognition site overlapping the ribosome binding site. They also identified a repressor protein RsmA that can recognize the same site, suggesting that RsmA is a downstream regulatory element of the GacA-GacS control system. A RsmA repressor protein was originally identified in E. carotovora and was found to regulate secondary metabolite synthesis and offI (AHF synthase) expression (4, 7); this protein was homologous to CsrA, which regulated carbon storage in E. coli (19, 21). CsrA and RsmA were found to bind to cognate regulatory RNA molecules; CsrB is a 350-nucleotide regulatory RNA identified in E. coli (20), and RsmB (previously AepH) is a 259-nucleotide regulatory RNA in E. carotovora (22). It is proposed that binding to CsrB and RsmB antagonizes the regulatory activity of CsrA and RsmA, respectively. This mechanism of RNA-protein interaction has not, as yet been described in Pseudomonas species; however, the recent identification of an RsmA homologue in P. fluorescens CHAO suggests that this regulatory mechanism may exist.

In this study, the secondary structure of the prrB RNA was generated by mfold software (Fig. 2B). The structure is noteworthy for eight stem-loops with the most striking feature being the presence of imperfect 5′-AGGA-3′ repeats found predominantly in the ends of hairpin loops distributed throughout the RNA molecule and in single-stranded regions between the hairpins. This structure is similar to the regulatory RNA molecules RsmB and CsrB. It is noteworthy however, that although the secondary structure of PrrB RNA is similar to RsmB, there is little nucleotide sequence homology. Furthermore, primer extension, Northern, and sequence analyses suggested the size of the PrrB RNA molecule to be approximately 130 bases, considerably smaller than RsmB. Nevertheless, the structural similarity of PrrB with CsrB and RsmB suggests that PrrB may function in F113 in a mechanism similar to RsmB through abrogating the action of an as yet unidentified repressor of secondary metabolite synthesis. The high similarity between sequences of the repressor molecule RsmA, recently isolated from P. fluorescens CHAO (2), and CsrA (E. coli) (21) and RsmA (E. carotovora) (7) suggest that PrrB RNA is likely to interact with a RsmA-like molecule in F113.

It was interesting to note the presence of a consensus KdgR_{bac} recognition sequence upstream of the prrB transcription start site and a KdgR_{esc} site within the coding region of prrB. Extensive work in E. carotovora, E. chrysanthemi, and E. coli has established that KdgR is a general repressor of genes involved in pectinolysis, pectinase secretion, and also other genes including rsmB (23, 25). Our finding suggests that an as yet unidentified gene product similar to KdgR could negatively regulate prrB expression in P. fluorescens F113. If this were true, it would be prudent to investigate if KdgR is also involved in the GacA-GacS regulatory cascade.

ACKNOWLEDGMENTS

Simon Aarons and Abdelhamid Abbas contributed equally to this work.

We thank Mary O’Connell-Motherway, Pat Higgins, and Liam Burgess for advice and technical assistance.

This work was supported in part by grants awarded by the Irish Health Research Board (to F.O. and S.A.), the Higher Education Authority (HEA) (to F.O.), the Irish Science and Technology Agency Forbairt (to F.O.), and the European Commission (BIO4-CT96-0027, BIO4-CT96-0018, FMRX-CT96-0039, BIO4-CT97-2227, and BIO4-CT98-0248).

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