The global regulator ANR is essential for *Pseudomonas chlororaphis* strain PA23 biocontrol

Munmun Nandi,1 Carrie Selin,2 Gabriel Brawerman,3 W. G. Dilantha Fernando2 and Teresa R. de Kievit1

1Department of Microbiology, University of Manitoba, Winnipeg, Manitoba, Canada
2Department of Plant Science, University of Manitoba, Winnipeg, Manitoba, Canada
3Department of Pharmacology and Therapeutics, University of Manitoba, Winnipeg, Manitoba, Canada

*Pseudomonas chlororaphis* PA23 is a biocontrol agent capable of protecting canola from stem rot disease caused by the fungus *Sclerotinia sclerotiorum*. The focus of the current study was to elucidate the role of the transcriptional regulator ANR in the biocontrol capabilities of this bacterium. An anr mutant was created, PA23anr, that was devoid antifungal activity. In other pseudomonads, ANR is essential for regulating HCN production. Characterization of PA23anr revealed that, in addition to HCN, ANR controls phenazine (PHZ), pyrrolnitrin (PRN), protease and autoinducer (AHL) signal molecule production. In gene expression studies, hcnA, phzA, prnA and phzI were found to be downregulated, consistent with our endproduct analysis. Because the phenotype of PA23anr closely resembles that of quorum sensing (QS)-deficient strains, we explored whether there is a connection between ANR and the PhzRI QS system. Both phzl and phzR are positively regulated by ANR, whereas PhzR represses anr transcription. Complementation of PA23anr with pUCP-phzR, C6-HSL or both yielded no change in phenotype. Conversely, PA23phzR harbouring pUCP23-anr exhibited partial-to-full restoration of antifungal activity, HCN, PRN and AHL production together with hcnA, prnA, phzI and rpoS expression. PHZ and protease production remained unchanged indicating that ANR can complement the QS-deficient phenotype with respect to some but not all traits. Our experiments were conducted at atmospheric O2 levels underscoring the fact that ANR has a profound effect on PA23 physiology under aerobic conditions.

INTRODUCTION

Certain pseudomonads are able to inhibit fungal pathogens via the production of secondary metabolites through a process known as biological control. *Pseudomonas chlororaphis* strain PA23 is able to suppress canola stem rot caused by the fungal pathogen *Sclerotinia sclerotiorum* in both greenhouse and field studies (Savchuk & Dilantha Fernando, 2004; Fernando et al., 2007). A number of compounds contribute to the disease-suppressive ability of this bacterium, including pyrrolnitrin (PRN), phenazine (PHZ), hydrogen cyanide (HCN), proteases, lipases and siderophores (Poritsanos et al., 2006; Zhang et al., 2006). Multiple regulatory elements oversee production of these metabolites at the transcriptional and posttranscriptional level. For example, the Gac-Rsm system, the stationary phase sigma factor RpoS, a positive activator of rpoS, called PsrA and the stringent response (SR) all function to control expression of antifungal (AF) compounds (Poritsanos et al., 2006; Manuel et al., 2012; Selin et al., 2012, 2014). In addition, biocontrol compounds are expressed in a population-density-dependent fashion under control of the Phz quorum sensing (QS) system (Selin et al., 2012). The transcriptional regulator PhzR becomes activated upon binding to its cognate acyl-homoserine lactone (AHL), C6-HSL, allowing it to control transcription of target genes. Adding to the complexity of this multi-tiered regulatory cascade is the fact that substantial cross-regulation exists between the regulators themselves. rpoS is subject to positive control by QS and the SR, while RpoS and the SR exert inductive and repressive effects on phzl and phzR,
respectively (Selin et al., 2012). Expression of rsmZ, rsmA and rsmE is similarly governed by several of the aforementioned regulators (Selin et al., 2014).

In Pseudomonas aeruginosa (Pessi & Haas, 2000) and Pseudomonas protegens CHA0 (Laville et al., 1998), a global regulator, called ANR (anaerobic regulator), has been shown to control hcnABC transcription and hydrogen cyanide (HCN) production. ANR is an FNR-like (gumarate and gitterate reductase regulator) transcriptional regulator belonging to the FNR-CRP (εAMP receptor protein) superfamily (Körner et al., 2003; Spiro, 1994). First discovered in Escherichia coli, these regulators can respond to various stimuli, including oxygen, nitric oxide, carbon monoxide and cyclic nucleotides (Körner et al., 2003; Lambden & Guest, 1976). Studies on P. aeruginosa have shown that, below a threshold concentration of O₂, ANR multimerizes via a dimerization helix (Sawers, 1991; Yoon et al., 2007). The dimeric form of this protein carries a [4Fe-4S]²⁺ cluster which acts as a cofactor for ANR (Spiro, 1994). Three N-terminal cysteine residues and one internal cysteine residue (Cys-20, Cys-23, Cys-29 and Cys-122) are predicted to bind to the [4Fe-4S]²⁺ cluster at O₂ concentrations below 5 µM (2.5 % air saturation). Above this O₂ level, the [4Fe-4S]²⁺ cluster is converted to [2Fe-2S]²⁺ which reportedly leads to ANR inactivation (Jordan et al., 1997; Zimmermann et al., 1991). In its active state, ANR binds to a conserved sequence (5’-TTGATNNNNATCAA-3’) referred to as an anr box in the promoter region of target genes (Yoon et al., 2007; Winteler & Haas, 1996).

ANR is believed to play a key role in allowing bacteria to adapt to microaerophilic or anaerobic conditions. In P. aeruginosa, ANR controls genes involved in energy production under low O₂ conditions, such as those encoding denitrification pathways, high-affinity cytochrome oxidases and arginine fermentation enzymes (Zimmermann et al., 1991; Ye et al., 1995). Moreover, optimal expression of the HCN synthase depends upon ANR and occurs at reduced O₂ levels in both P. aeruginosa and P. protegens (Castric, 1983, 1994). In the former, ANR functions together with its two QS systems, Las and Rhl, to control hcnABC transcription (Pessi & Haas, 2000). Intriguingly, there appears to be a substantial degree of overlap between the QS and ANR regulators in P. aeruginosa (Hammond et al., 2015; Schuster & Greenberg, 2006). ANR binding consensus was identified in 25 % of QS-controlled promoters, underscoring that this dual regulation extends well beyond the hcnABC operon (Schuster & Greenberg, 2006). Whereas for P. protegens CHA0, which lacks an AHL-based QS system, ANR is the sole regulator controlling hcn transcription (Laville et al., 1998; Blumer & Haas, 2000). Under water-saturated soil conditions, a CHA0 anr mutant exhibited a 30 % decrease in disease suppression highlighting the importance of this regulator in soils with restricted oxygen availability (Laville et al., 1998). There is also evidence to support a role for this regulator under aerobic conditions. In Pseudomonas putida KT2440, ANR positively regulates expression of the Chb3-1 terminal oxidase during exponential growth in highly aerated medium (Ugidos et al., 2008).

At present, with the exception of P. protegens CHA0 (Laville et al., 1998), there is little known about the impact of ANR on the AF activity of pseudomonads. The aim of the current investigation was to explore the role of the global regulator ANR in the biocontrol capability of P. chlororaphis strain PA23. We discovered that, in addition to HCN, production of PRN, PHZ, AHL signals and protease activity are all subject to ANR regulation. Furthermore, we show that ANR and QS exhibit cross-regulation. ANR positively controls expression of phzl and phzR, whereas PhzR functions as a repressor of anr transcription.

**METHODS**

**Bacterial strains and growth conditions.** Bacterial strains and plasmids used in this study are listed Table 1. E. coli was cultured at 37 °C on Lysogeny Broth agar (Difco Laboratories, Detroit, MI, USA). P. chlororaphis strains were cultured on Lysogeny Broth medium at 28 °C or in M9 minimal salts medium amended with 0.2 % glucose and 1 mM magnesium sulfate (MgSO₄). Unless otherwise indicated, vigorous aeration of cells was achieved by shaking 10 ml culture volumes in 125 ml flasks at 210 r.p.m. Antibiotics were used at the following concentrations: ampicillin (Amp; 100 µg ml⁻¹), chloramphenicol (Cm; 12.5 µg ml⁻¹), gentamicin (Gm; 15 µg ml⁻¹) and tetracycline (Tc; 15 µg ml⁻¹) for E. coli and piperacillin (Pip; 40 µg ml⁻¹), Gm (20 µg ml⁻¹) and Tc (15 µg ml⁻¹) for Pseudomonas strains. Antibiotics were obtained from Research Products International (Mount Prospect, IL, USA).

**Nucleic acid manipulation.** Standard techniques were employed for purification, cloning and other manipulations of DNA (Sambrook et al., 1989). PCR was performed following standard conditions suggested by Thermo Fisher Scientific data sheets supplied with their Taq polymerase.

**Generation of PA23anr and PA23phzRanr mutants.** Primers used for the construction of the mutants are listed in Table 1. To generate PA23anr, an internal region of the anr gene cluster was PCR amplified using primers anr-FOR and anr-REV. Primers were designed based on the sequence of the anr gene obtained from P. chlororaphis PA23 (GenBank accession no. NZ_CP000896). The 726 bp PCR amplicon was cloned into pCR2.1, generating pCRanr-23. To liberate the insert, pCRanr-23 was digested with HindIII and XbaI and was subcloned into the same sites of the pKNOCK-Tc suicide vector. Triparental mating among the donor [E. coli DH5α Apir (pKNOCK-Tc)], helper [E. coli DH5α (pRK6000)] and recipient (PA23phzR) was performed to insert an anr 23. The 959 bp PCR product was used to screen for transconjugants. To create a PA23phzRanr double mutant, triparental mating was performed among the donor [E. coli DH5α Apir (pKNOCK-Tc)], helper [E. coli DH5α (pRK6000)] and recipient (PA23phzR) strains. The anr mutation in PA23anr and PA23phzRanr was confirmed by PCR analysis using anr-FOR/tet-REV and anr-REV/tet-FOR primers.

**Plasmid construction.** For PA23anr complementation, a 959 bp PCR fragment containing the entire anr gene was obtained using anr(comp)-FOR and anr(comp)-REV. The amplicon was cloned into pCR2.1 to yield pCRanr(comp)–23. The 959 bp SacI–XbaI fragment was excised from pCRanr(comp)–23 and subcloned into the same sites of pUCP23, creating pUCP23-anr.

**AF assay.** The AF activity of PA23 and derivative strains against S. scelotitum was assessed through in vitro radial diffusion assays on one-fifth potato dextrose agar medium as described previously (Poritsanos et al., 2012).
et al., 2006). Five replicates were analysed for each strain and the experiments were repeated three times.

**Exoproduct analyses.** Protease activity and the production of AHL molecules were analysed according to Poritsanos et al. (2006). Data represent the average of five replicates and assays were repeated three times.

**Qualitative HCN analysis.** HCN production was monitored using Cyantesmo paper (Macherey-Nagel, Germany), which turns blue in the...
Quantitative analysis of PHZ and PRN production. Bacterial cultures (15 ml) were grown in M9 minimal medium supplemented with 0.2% glucose and 1 mM MgSO\(_4\) at 28°C in 250 ml shake flasks at 210 r.p.m. Cultures were allowed to grow for 20 h before being subjected to PHZ analysis (Selin et al., 2010). PRN extractions were performed after 5 days of growth and quantified by HPLC according to previously described methods (Selin et al., 2010). Samples were analysed in triplicate and the experiments were repeated twice.

Gene expression analysis using quantitative real-time PCR. To monitor expression of metabolite and regulatory genes involved in biocontrol, quantitative real-time PCR (qRT-PCR) was employed. The expression of prn, phz, hcn, phzR, anr, rpoB and rpoS was determined in PA23 and derivative strains. The housekeeping gene rpoB was chosen to normalize expression values of the target genes. Primers used for gene expression analysis are listed in Table 1. Primers were designed based on the sequences of the respective genes obtained from P. chlororaphis PA23 (GenBank accession no. NZ_CP008696). Cultures were grown for 24 h in M9 minimal medium (0.2% glucose and 1 mM MgSO\(_4\)) at 28°C. Total RNA was extracted from a 0.5 ml volume of culture using an RNeasy Mini Kit (QIAGEN, Valencia, USA) and residual genomic DNA was removed by treatment with TURBO RNase-free DNase I (Ambion, Carlsbad, USA). cDNA was generated by reverse transcription using the Maxima First-Strand cDNA Synthesis Kit (Thermo Scientific, Rockford, USA) and the following conditions were used:

<table>
<thead>
<tr>
<th>Strains</th>
<th>AF*</th>
<th>Protease*</th>
<th>Autoinducer*</th>
<th>HCN†</th>
<th>PHZ (µg ml(^{-1}))‡</th>
<th>PRN (µg ml(^{-1}))‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA23</td>
<td>5.6 (0.4)</td>
<td>6.6 (0.4)</td>
<td>7.4 (0.5)</td>
<td>+++</td>
<td>37.16 (3.59)†</td>
<td>2.80 (0.36)†</td>
</tr>
<tr>
<td>Δanr</td>
<td>0§</td>
<td>0§</td>
<td>0§</td>
<td>+</td>
<td>8.67 (3.87)§</td>
<td>0§</td>
</tr>
<tr>
<td>Δanr (anr-pUCP23)</td>
<td>4.8 (0.3)</td>
<td>6.2 (0.3)#</td>
<td>7.3 (0.4)#</td>
<td>+++</td>
<td>33.34 (4.06)#</td>
<td>2.70 (0.30)#</td>
</tr>
<tr>
<td>Δanr + C(_6)-HSL</td>
<td>0§</td>
<td>0§</td>
<td>3.0 (0.4)$§</td>
<td>+</td>
<td>7.94 (1.56)$§</td>
<td>0.05 (0.05)$§</td>
</tr>
<tr>
<td>Δanr (phzR-pUCP23)</td>
<td>0§</td>
<td>0§</td>
<td>0§</td>
<td>+</td>
<td>7.14 (2.80)$§</td>
<td>0.06 (0.05)$§</td>
</tr>
<tr>
<td>Δanr (phzR-pUCP23) + C(_6)-HSL</td>
<td>0§</td>
<td>0§</td>
<td>3.3 (0.5)$§</td>
<td>+</td>
<td>8.33 (2.64)$§</td>
<td>0.06 (0.05)$§</td>
</tr>
<tr>
<td>ΔphzR</td>
<td>0§</td>
<td>5.4 (0.4)$§</td>
<td>3.3 (0.4)$§</td>
<td>+</td>
<td>6.37 (1.51)$§</td>
<td>0§</td>
</tr>
<tr>
<td>ΔphzR (phzR-pUCP23)</td>
<td>4.3 (0.3)$§</td>
<td>5.7 (0.3)$§</td>
<td>4.8 (0.3)$§</td>
<td>++</td>
<td>32.06 (2.82)$§</td>
<td>2.25 (0.30)$§</td>
</tr>
<tr>
<td>ΔphzR (anr-pUCP23)</td>
<td>4.2 (0.3)$§</td>
<td>4.5 (0.3)$§</td>
<td>5.3 (0.2)$§</td>
<td>++</td>
<td>8.02 (1.98)$§</td>
<td>0.95 (0.18)$§</td>
</tr>
</tbody>
</table>

*Mean (sd) of the zones of activity (mm) obtained from five replicates.
†Determined using cyanescino paper. +, low; ++, intermediate; ++++, high.
‡Mean (sd) obtained from a triplicate set.
§Significantly different from WT (P<0.001).
¶Significantly different from WT (P<0.05).
$Significantly different from WT (P<0.01).
#Not significantly different from WT.
infection process. To infect the petals, fresh detached leaves were inoculated with ascospore-infected petals, on susceptible leaves of canola, simulating the infection process under natural conditions. The senescent leaves were soaked in bacterial suspensions (2×10^6 c.f.u. ml^-1) or water (positive control) for 2 min, followed by 40 cycles of 98°C for 30 s, 60°C for 30 s and 72°C for 30 s. The reactions were performed in triplicate and experiments were repeated three times with four biological replicates. Relative gene expression was determined using the ΔΔCt method as described by Livak & Schmittgen (2001).

Leaf infection assay. The WT PA23 and derivative strains were assessed for their efficiency in suppressing lesion formation by S. sclerotiorum on susceptible leaves of canola [Brassica napus (cv. Westar)]. Fresh detached leaves were inoculated with ascospore-infected petals, simulating the infection process under natural conditions. The senescent flower petals of canola were used as they facilitate the S. sclerotiorum infection process. To infect the petals, a S. sclerotiorum ascospore suspension (5×10^5 spores ml^-1) was prepared in 0.01% Tween-80 solution. Each petal was inoculated with a 30 µl droplet of ascospore suspension or 0.01 % Tween-80 (negative control). Petals were placed on a petriplate, which was sealed with parafilm to retain humidity and incubated at 22°C for 72 h. Lesion formation on the leaves was monitored over the course of 72 h. Six biological replicates were analysed for each strain and the assays were repeated three times.

Statistical analysis. An unpaired Student’s t-test was used for statistical analysis of PHZ, PRN and AHL production; AF activity; protease production; and gene expression using qRT-PCR.

RESULTS

Phenotypic characterization of PA23anr

An anr mutant of strain PA23 was generated and assessed for its ability to inhibit S. sclerotiorum in vitro. A complete loss of fungal antagonism was observed for PA23anr, which was restored to near WT levels upon complementation with anr-pUCP23 (Fig. 1, Table 2). In other pseudomonads, ANR positively regulates HCN production and, as expected, HCN expression was dramatically reduced in the anr mutant (Table 2). PA23 produces the diffusible antibiotics PHZ and PRN. PHZ imparts an orange colour to this bacterium and PRN is the primary antibiotic responsible for biocontrol (Selin et al., 2010). The lack of AF activity along with reduced pigmentation suggested that the anr mutant was producing decreased levels of these compounds (Fig. 1). Antibiotic quantification revealed that PHZ and PRN were markedly reduced in PA23anr (Table 2). Moreover, the anr mutant exhibited a complete loss of extracellular protease activity (Fig. 2, Table 2). As PHZ, PRN and...
protease production are under QS control (Selin et al., 2012), we were interested to determine whether QS is affected by an anr deficiency. In qualitative AHL assays, the Δanr strain showed a lack of AHL signal production (Fig. 3, Table 2). The modest purple zones surrounding the Δanr + C6-HSL and Δanr (phzR-pUCP23) + C6-HSL samples is due to the exogenous AHL added to the liquid cultures. Since these cells are not producing their own AHL, markedly less purple pigmentation results compared to AHL-secreting bacteria. As expected, PA23anr carrying a plasmid-borne copy of anr exhibited near WT levels of HCN, PRN, PHZ and AHL production (Fig. 3, Table 2). No differences in growth rate were observed between the WT and anr mutant (data not shown).

**ANR positively regulates QS, rpoS and antibiotic gene expression in PA23**

We employed qRT-PCR to monitor phzA, prnA, hcnA, phzI, phzR, rpoS and anr expression in PA23 and the Δanr strain. In the Δanr mutant, all of the aforementioned genes showed reduced expression indicating that ANR positively regulates these biosynthetic and regulatory genes (Fig. 4).

Typically, ANR binds to a conserved 14 nt sequence, known as the anr box, located in the promoter region of target genes (Yoon et al., 2007; Winteler & Haas, 1996). Inspection of the PA23 hcnA promoter region revealed the presence of a 14 nt anr box (163 bp from the ATG start), which is 100% identical with that found upstream of the P. protegens CHA0 hcnA gene (Laville et al., 1998) (Fig. 5a).

We next looked for anr boxes upstream of phzA, prnA, phzI, phzR, rpoS and anr. Sequences having 8–10 nt in common with the anr box consensus of hcnA were identified upstream of phzA (69 bp from the ATG start), prnA (264 bp from the ATG start), phzI (99 bp from the ATG start) and phzR (45 bp from the ATG start) (Fig. 5a). Similarly, an anr box was identified in the promoter regions of rpoS (74 bp from the ATG start; 8/14 bp match) and anr (268 bp from the ATG start; 10/14 bp match) (Fig. 5a). The presence of an anr box together with the observed decrease in anr expression in PA23anr indicates that this protein is subject to positive autoregulation.

**PhzR negatively regulates anr expression**

Since ANR positively regulates both the phzI and phzR QS genes, we were interested to determine whether there is reciprocal cross-regulation of anr by QS. qRT-PCR analysis was conducted to monitor anr, as well as phzA, prnA, hcnA, phzI, phzR and rpoS transcription in the ΔphzR strain. With the exception of anr, transcription of all of the genes was markedly reduced in the phzR mutant background (Fig. 4). A greater than twofold increase in anr transcription was observed in the phzR mutant, suggesting that PhzR has a repressive effect on anr (Fig. 4).
**ANR is essential for *P. chlororaphis* PA23 biocontrol**

To determine whether constitutively expressed *anr* in *trans* would complement the *phzR* mutant, PA23*phzR* (*anr*-pUCP23) was characterized. We discovered that *anr*-pUCP23 was able to fully or at least partially restore AF activity, as well as AHL, HCN and PRN production in the *phzR* mutant (Figs 1 and 3, Table 2). Conversely, PHZ production and protease activity remained unchanged (Table 2). qRT-PCR analysis was conducted to assess gene expression in the Δ*phzR* strain harbouring *anr*-pUCP23. Expression of *hcnA*, *prnA*, *phzI* and *rpoS* was elevated but remained below that of WT levels (Fig. 4), consistent with our phenotypic findings (Table 2). However, the *phzR* mutant harbouring *anr*-pUCP23 showed no change in *phzA* expression, which correlates with the decreased PHZ levels observed for this strain (Fig. 4, Table 2). While unaltered *phzR* expression was observed in this background, *anr* expression was significantly increased further establishing that ANR is subject to positive autoregulation (Fig. 4).

**DISCUSSION**

The aim of the current investigation was to explore the role of the global regulator ANR in the biocontrol capability of *P. chlororaphis* strain PA23. In order to suppress stem rot of canola, PA23 is applied to the aerial surfaces of the plant where *Sclerotinia* infection takes place (Poritsanos et al., 2006; Zhang, 2004). Accordingly, all of our laboratory experiments were conducted at atmospheric O$_2$ levels,
consistent with conditions in the phyllosphere. Characterization of an anr mutant revealed that the absence of this regulator leads to a total loss of AF activity; thus, ANR has a profound effect on PA23 physiology.

In P. protegens CHA0, ANR controls HCN synthesis, whereas 2,4-diacetylphloroglucinol and pyoluteorin production are unaffected by an anr deficiency (Laville et al., 1998). We discovered that the phenotype of a PA23 anr mutant is dramatically more pronounced than what is observed for CHA0. Besides HCN, production of PRN and PHZ, protease activity and QS were markedly decreased in a PA23 anr-deficient background. Gene expression profiles show significant downregulation of biosynthetic and regulatory genes in keeping with our endproduct analyses (Fig. 4).

One of the striking features of the anr mutant is its close resemblance to that of PA23 phzR. Both mutants exhibited a complete loss of fungal antagonism and reduced exometabolite production (Table 2). In addition, reduced expression of hcnA, prnA, phzA, phzI and rpoS was observed in PA23anr and PA23phzR (Fig. 4). Taken together, our phenotypic and gene expression analysis indicate that ANR and QS are both important for the production of secondary metabolites in PA23. Schuster & Greenberg (2006) reported similar findings for P. aeruginosa wherein an anr box sequence was identified in the promoter regions of 25% of QS-controlled genes. In another study, ANR was found to control expression of multiple QS-associated genes in P. aeruginosa strains PAO1 and J215 (Hammond et al., 2015). Thus, in P. aeruginosa, there appears to be significant overlap between the QS and ANR regulons.

To further explore connections that exist between QS and ANR, we investigated whether these two regulatory systems are capable of cross-regulation. We discovered that PhzR has a repressive effect on anr with expression up to fourfold in the ΔphzR background (Fig. 4). Hammond et al. (2015) reported that the LasRI QS system acts as a repressor for ANR in P. aeruginosa strains PAO1 and J215 consistent with findings presented herein. We also discovered that ANR positively controls QS in PA23 with phzR and phzI expression and C6-HSL synthesis all dramatically reduced in an anr mutant background (Figs 2 and 4). The fact that ANR positively regulates QS raises the possibility that the effect of ANR on PA23

Fig. 6. Detached leaf infection assay to assess the efficiency of PA23 and derivative strains in suppressing S. sclerotiorum lesions on leaves of canola [Brassica napus (cv. Westar)]. Note that, in the absence of either ANR or PhzR, PA23 is unable to suppress S. sclerotiorum lesion formation. However, plasmid-borne anr restores the biocontrol potential of both the anr- and phzR-deficient strains. Six biological replicates were analysed for each strain and the assays were repeated three times. One representative data set is shown.
biocontrol is indirectly mediated through QS. Complementing PA23<sup>anr</sup> with <i>phzR</i>-pUCP23, exogenous C<sub>6</sub>-HSL or both yielded no change in gene expression or exoproduct formation (Table 2, Fig. 4). QS alone is therefore insufficient to activate target gene expression in an <i>anr</i> mutant background. On the other hand, complementing a <i>phzR</i> mutant with <i>anr</i>-pUCP23, exogenous C<sub>6</sub>-HSL or both yielded no change in gene expression or exoproduct formation (Table 2, Fig. 4). QS alone is therefore insufficient to activate target gene expression in an <i>anr</i> mutant background. On the other hand, complementing a <i>phzR</i> mutant with <i>anr</i>-pUCP23 resulted in partial restoration of <i>hcnA</i>, <i>prnA</i>, <i>phzI</i> and <i>rpoS</i> expression, together with AF activity, HCN, PRN and AHL production (Table 2, Fig. 4). Only <i>phz</i> expression, PHZ production and protease activity remained unchanged (Table 2, Fig. 4). Thus, providing <i>anr</i> in trans can partially complement the QS-deficient phenotype in terms of some but not all biocontrol genes and products. In <i>P. aeruginosa</i>, <i>ANR</i> can in part substitute for a loss of QS with respect to virulence factor regulation, consistent with findings presented herein (Hammond <i>et al.</i>, 2015).

<sup>Fig. 7.</sup> Schematic model of promoter recognition by the transcriptional regulators ANR and PhzR-C<sub>6</sub>-HSL through their interaction with the αCTD (C-terminal domain of the alpha subunit) and αNTD (N-terminal domain of the alpha subunit) of RNA polymerase, shown as separate domains joined by a linker. (a) <i>anr</i> and <i>phz</i> boxes in close proximity, consistent with the 23–45 bp intervening region found within the <i>hcnA</i>, <i>prnA</i>, <i>phzI</i> and <i>rpoS</i> promoters. In this case, either ANR or PhzR-C<sub>6</sub>-HSL is sufficient for activation of gene expression. In (b), the two binding sequences are further apart, reflective of the 99 bp separation observed for <i>phzA</i>. In this configuration, both ANR and PhzR-C<sub>6</sub>-HSL are required for gene expression. This model has been modified from that proposed by Pessi & Haas (2000).
region separating the phz and anr boxes implies that both regulators are required to activate the phz biosynthetic operon. In Fig. 7, we present a model of QS and ANR activation of the aforementioned genes. At this point, the proposed regulatory scheme remains speculative; DNA binding studies are required to clarify whether ANR and PhzR-AHL bind separately or cooperatively at these promoter regions. It is intriguing that phz operon expression is dependent upon both regulators since the encoded products have multiple functions beyond their inhibitory effects (Price-Whelan et al., 2006). For example, PHZs have been shown to contribute to rhizosphere persistence of biocontrol pseudomonads (Mazzola et al., 1992) possibly through facilitating biofilm formation on roots. Additional established or proposed roles for these molecules include cellular energy generation, maintenance of redox homeostasis and iron acquisition (Hernandez & Newman, 2001; Hernandez et al., 2004; Price-Whelan et al., 2006). In P. aeruginosa, the PHZ pyocyanin is not only regulated by QS but it also acts as an intercellular QS signal itself (Dietrich et al., 2006). As these antibiotics affect diverse aspects of bacterial physiology, strict control over their production may be required.

For the most part, FNR-like transcriptional regulators are believed to function optimally under low O2 concentrations. In P. aeruginosa PAO1, for example, ANR is essential for anaerobic growth mediated by both denitrification and arginine deiminase pathways (Galimand et al., 1991; Alvarez-Ortega & Harwood, 2007). There are reports showing that the role of these regulators is not restricted to anaerobic conditions. In P. putida KT2440, ANR activates expression of the Cbb3-1 terminal oxidase during growth under highly aerated conditions (Ugidos et al., 2008). A recent study focused on this same strain identified three FNR proteins with different sensitivities to O2 (Ibrahim et al., 2015). Comparison with the FNR of E. coli revealed deviations in the amino acid residues close to four conserved cysteines in the P. putida FNR proteins. Two out of the three FNRs had higher mismatches and were found to be less sensitive to O2 (Ibrahim et al., 2015). Moreover, it has been established that, in E. coli, replacing specific amino acids adjacent to the cysteine residues can increase the stability of the [4Fe-4S] cluster as well as FNR activity in the presence of O2 (Bates et al., 2000; Jervis et al., 2009). Based on sequence alignment with E. coli FNR, the PA23 ANR protein is 53% identical (173/226 similar amino acid residues), with three N-terminal cysteine residues and one internal cysteine residue conserved between them (Fig. S1, available in the online Supplementary Material). Importantly, we found mismatches adjacent and close to the N-terminal cysteine residues, which may contribute to the high degree of functionality observed for PA23 ANR in the presence of O2 (Fig. S1).

In summary, ANR is well established as being required for HCN synthesis in P. aeruginosa and P. protegens CHA0 and contributes to the biocontrol capacity of the latter (Laville et al., 1998). In the current study, we discovered that ANR is essential for expression of not only HCN but also of a plethora of secreted products including PHZ and PRN, degradative enzymes and AHL molecules. All of our analysis was carried out on cells grown in highly aerated media underscoring the fact that ANR regulates multiple aspects of PA23 physiology under aerobic conditions. To the best of our knowledge, this is the first report of ANR having such a pronounced effect on the biocontrol capacity of a pseudomonad.

REFERENCES


The transcriptional activators LasR and RhlR (VsmR) to expression of the biofilm.


Laville, J., Blumer, C., Von Schroetter, C., Gaia, V., D...