Iron regulation of the *hcnABC* genes encoding hydrogen cyanide synthase depends on the anaerobic regulator ANR rather than on the global activator GacA in *Pseudomonas fluorescens* CHA0

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***Pseudomonas fluorescens* CHA0 produces hydrogen cyanide (HCN), a secondary metabolite that substantially contributes to this strain's biocontrol ability. Cyanogenesis is induced by oxygen-limiting conditions, but abolished by iron depletion. In *P. fluorescens*, the anaerobic regulator ANR and the global activator GacA are both required for the maximal expression of the HCN biosynthetic genes *hcnABC*. The molecular basis of this regulation by ANR and GacA was investigated under conditions of oxygen and iron limitation. A promoter deletion analysis using a translational *hcnA'*–*lacZ* fusion revealed that a conserved FNR/ANR recognition sequence in the –40 promoter region was necessary and sufficient for the regulation by ANR in response to oxygen limitation. Stimulation of *hcnA'*–*lacZ* expression by the addition of iron also depended on the presence of ANR and the FNR/ANR box, but not on GacA, suggesting that in addition to acting as an oxygen-sensitive protein, ANR also responds to iron availability. Expression of the translational *hcnA'*–*lacZ* fusion remained GacA-dependent in *hcn* promoter mutants that were no longer responsive to ANR, in agreement with earlier evidence for a post-transcriptional regulatory mechanism under GacA control. These data support a model in which cyanogenesis is sequentially activated by ANR at the level of transcription and by components of the GacA network at the level of translation.**

Keywords: *Pseudomonas fluorescens*, hydrogen cyanide, ANR, GacA, iron regulation

INTRODUCTION

The biosynthesis of the secondary metabolite hydrogen cyanide (HCN) has been demonstrated in a small number of bacterial species, such as *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* and *Chromobacterium violaceum* (Askeland & Morrison, 1983; Castric, 1975; Knowles & Bunch, 1986). Cyanogenesis is maximal during the transition from exponential to stationary phase (Castric *et al.*, 1979; Askeland & Morrison, 1983) and is influenced by several environmental factors including iron, phosphate and

oxygen concentrations (Knowles & Bunch, 1986). Iron has a stimulatory effect on cyanogenesis in all three of these bacterial species (Askeland & Morrison, 1983; Castric, 1975; Rodgers & Knowles, 1978; Voisard *et al.*, 1989). Askeland & Morrison (1983) demonstrated a linear relationship between HCN production and the log of iron concentration over a range of 3 to 300 µM in *P. fluorescens*. In batch cultures of *P. aeruginosa* and *P. fluorescens*, HCN production is optimally induced when oxygen is growth-limiting (Castric, 1983; Laville *et al.*, 1998). HCN synthase, the enzyme producing HCN and CO₂ from glycine (Wissing, 1975; Castric 1977), transfers four electrons to the respiratory chain, with molecular oxygen as the terminal acceptor. At the same time, the enzyme is very sensitive to molecular oxygen and as a consequence becomes rapidly inactivated in the

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presence of oxygen (Castric, 1981, 1983, 1994). Whilst the role of HCN in bacterial physiology remains obscure, an ecological role for bacterial cyanogenesis has been discovered in the case of the root-colonizing and plant-beneficial *P. fluorescens* strain CHA0 (Voisard *et al.*, 1989), which protects several plants from fungal root diseases (Voisard *et al.*, 1994; Schnider *et al.*, 1995). HCN production by strain CHA0 accounts for part of the strain's biocontrol capacity, for example the suppression of tobacco black root rot caused by *Thielaviopsis basicola* (Voisard *et al.*, 1989; Laville *et al.*, 1998). Iron sufficiency is important for both HCN production and disease suppression (Keel *et al.*, 1989; Voisard *et al.*, 1989).

HCN synthase of *P. fluorescens* CHA0 is encoded by the *hcnABC* gene cluster (Laville *et al.*, 1998). Two regulatory proteins have been demonstrated to control cyanogenesis in this bacterium: the global activator GacA and the anaerobic regulator ANR (Laville *et al.*, 1992, 1998). The same regulatory elements are also involved in HCN formation in *P. aeruginosa* (Reimann *et al.*, 1997; Zimmermann *et al.*, 1991). GacA, the response regulator of the two-component regulatory system GacS (formerly LemA)/GacA, is required not only for the synthesis of HCN, but also for that of other secondary metabolites and exoenzymes in strain CHA0 (Laville *et al.*, 1992; Sacherer *et al.*, 1994). GacA activates a regulatory network in which the RNA-binding protein RsmA is one of the downstream elements. The region of translation initiation of the *hcnA* gene is important for regulation by GacA and RsmA (Blumer *et al.*, 1999; Blumer & Haas, 2000). The GacS/GacA system is conserved in many Gram-negative bacteria. In plant-beneficial strains of *P. fluorescens* and *Pseudomonas aureofaciens*, this system regulates biocontrol activity (Laville *et al.*, 1992; Gaffney *et al.*, 1994; Chancey *et al.*, 1999). In plant- and animal-pathogenic species of *Pseudomonas*, *Erwinia*, *Vibrio* and *Salmonella*, GacA function is required for virulence (Rich *et al.*, 1994; Erickson *et al.*, 1998; Wong *et al.*, 1998; Rakeman & Miller, 1999; Kinscherf & Willis, 1999). The signals interacting with the GacS sensor are unknown.

Mutants of both *P. fluorescens* and *P. aeruginosa* lacking the anaerobic regulator ANR, an FNR homologue, are defective in HCN biosynthesis (Laville *et al.*, 1998; Zimmermann *et al.*, 1991). FNR of *Escherichia coli* is a dimeric transcriptional activator which, by virtue of two bound $[4\text{Fe-4S}]^{2+}$ clusters, senses intracellular oxygen concentrations (Kiley & Beinert, 1999). Below a threshold concentration (about 10 μM) of dissolved oxygen, FNR can activate or repress transcription of target genes by binding to a conserved sequence known as the FNR box (Spiro, 1994; Rhodius & Busby, 1998). The ANR protein of *P. fluorescens* CHA0 is structurally and functionally very similar to FNR (Laville *et al.*, 1998; Højberg *et al.*, 1999). The transcription start site of the *hcnABC* gene cluster of *P. fluorescens* has been mapped (Fig. 1). The *hcn* promoter

Fig. 1. Structure of the *hcnABC* operon of *P. fluorescens* CHA0 and sequence of the promoter region. +1, Transcription start site; EcoRI*, artificially introduced site in pME3225. The sequence absent in pME3223 is indicated by two vertical bars.

contains a typical FNR/ANR box in the -40 region (Laville *et al.*, 1998). Moreover, a translational *hcnA'*-*lacZ* fusion is strongly induced under oxygen-limited conditions in the wild-type, whereas little expression occurs in an *anr* mutant (Laville *et al.*, 1998).

ANR and GacA do not regulate the expression of each other in *P. fluorescens* (Blumer *et al.*, 1999), but they might synergistically interact during activation of cyanogenesis. The aims of this study were to determine whether such an interaction occurs at the *hcn* promoter and to find out whether the stimulatory effect of iron on HCN synthesis is mediated by one of these regulators.

METHODS

Bacterial strains and growth conditions. *P. fluorescens* strains used were the wild-type CHA0 and its derivatives CHA89 (*gacA*::Km^R) and CHA21 (*anr*::Km^R) (Laville *et al.*, 1992, 1998). *P. aeruginosa* PAO1 (wild-type) and PAO1A4 (*fur*-4) (Barton *et al.*, 1996) were used to assess iron regulation by Fur. Bacterial strains were routinely grown in nutrient yeast broth (NYB) or on nutrient agar plates (Stanisich & Holloway, 1972) at 30 °C (*P. fluorescens*) or at 37 °C (*E. coli*, *P. aeruginosa*). When required, tetracycline was added to the media at a concentration of 25 $\mu\text{g ml}^{-1}$ (*E. coli*) or 125 $\mu\text{g ml}^{-1}$ (*P. fluorescens*, *P. aeruginosa*). Clumping of the cells was reduced by the addition of 0.05 % Triton X-100 to the media. For aerobic growth, *P. fluorescens* CHA0 was cultured in baffled 500 ml Erlenmeyer flasks containing 60 ml NYB with vigorous shaking. Severely oxygen-limited ('anaerobic') cultures of *P. fluorescens* CHA0 were grown in rubber-stoppered 125 ml bottles with gentle shaking. The oxygen initially present in the medium was consumed by the strictly aerobic cells (Højberg *et al.*, 1999); growth stopped at an OD₆₀₀ of ≤ 1.0 because of oxygen limitation. Mild oxygen limitation occurred when cells were grown in 50 ml flasks containing 20 ml NYB with gentle shaking. In most experiments, cells were harvested at an OD₆₀₀ of 0.8–1.0 (determined in a Pharmacia Ultrospec III spectrophotometer) corresponding to about 10^9 cells ml⁻¹ from both aerobic and anaerobic cultures and used for β -galactosidase activity measurements. A synthetic minimal medium (MMC) described by Castric (1975) with or without 20 μM FeCl₃ was used to study the

Table 1. Nucleotide sequences of the primers used for mutagenesis of the *hcn* promoter region of *P. fluorescens*

Primer	Resulting construct	Sequence*
Primer 3 (<i>lacZ</i>)	All†	5'-TGCTGCAAGGCGATTAAGTTGG-3'
Primer 1	pME3219, pME3226	5'-GCTCGAATTCATGCGTCATTACTCTT-3'
Primer 2	pME3226	5'-CATGCAAGCTTCATCCGTGAAAAATGAATG-3'
Primer 7	pME3223	5'-GCTCGAATTCGGTGTCCGACAAACCGAC-3'
Primer 8	pME3225	5'-GCTCGAATTCCTGGCCCAGATCAATATCC-3'
Primer 9	pME3226	5'-CAAACCGACTATAATGGCCCGCGC-3'

* Additional bases at the 5' end that are not homologous to the *hcn* sequence are shown in bold face. Restriction sites (*Eco*RI, *Hind*III) are underlined. Double underlining indicates the mutations carried by primer 9.

† See Figs 2 and 3 for constructs. Primer 3 anneals at position 66–87 in the *lacZ* gene.

regulation of the *hcn* genes by iron. Cultures were grown in 50 ml flasks containing 20 ml MMC with shaking. An inoculum of $\sim 4 \times 10^8$ cells was used for 20 ml MMC. Under these conditions, cells were mildly oxygen-limited. Traces of iron present in the medium without FeCl_3 amendment allowed bacterial growth to an OD_{600} of ≤ 1.0 .

DNA manipulation. Small-scale preparations of plasmid DNA were carried out by the CTAB (cetyltrimethylammonium bromide) method (Del Sal *et al.*, 1988) for *E. coli* and by the alkaline lysis method (Sambrook *et al.*, 1989) for *P. fluorescens*. Large-scale preparation of plasmid DNA was performed using Qiagen-tip 100 columns. Transformation of *E. coli* and *P. fluorescens* strains was done by the standard CaCl_2 procedure (Sambrook *et al.*, 1989) or by electroporation (Farinha & Kropinski, 1990). DNA cloning experiments were carried out according to standard methods (Sambrook *et al.*, 1989). DNA fragments were generated by PCR as described previously (Laville *et al.*, 1998). All PCR and linker constructs were verified by nucleotide sequencing using the chain termination method with the Sequenase 2.0 kit (US Biochemical) and T7 polymerase (Pharmacia) following the manufacturers' instructions.

Construction of plasmids. Recombinant plasmids were constructed in the broad-host-range vector pME6010 (Heeb *et al.*, 2000). The translational *hcnA'*-*lacZ* fusion in pME3219 has been described previously (Laville *et al.*, 1998). Plasmids pME3223 and pME3225 were obtained by PCR-amplifying different parts of the *hcn* promoter region, using internal *Eco*RI-tagged primers which anneal to different sequences in the 5' region of the *hcn* promoter and an external primer (primer 3) which anneals within the *lacZ* sequence (Table 1). PCR products were generated from pME3219 as the template, restricted with *Eco*RI and *Pst*I and cloned into pME3219 previously cut with the same enzymes. The two-base substitution in the -10 region of the *hcn* promoter in plasmid pME3226 (Fig. 2) was generated by the overlap extension method (Mikaelian & Sergeant, 1996), using as flanking oligonucleotides primers 1, 2 (Laville *et al.*, 1998) and 3, together with the mutagenic primer 9 (Table 1). Plasmid pME3224 is identical to pME3219 except for a 17 bp *Ava*II deletion in the 5' untranslated region of the *hcn* genes (Fig. 3). For the construction of plasmid pME3238, a DNA linker with a 5' *Eco*RI and a 3' *Mae*I site carrying the ANR box (underlined) and a random downstream sequence (bold face) not homologous to the native *hcn* promoter sequence (5'-AATTCCTTGGCCCAGATCAACGCTTACACTGGTAGCGTTAGC-3') was synthesized. This linker was fused to a

*Mae*I/*Pst*I fragment containing the authentic -10 region and the beginning of the *hcnA* gene (Laville *et al.*, 1998) and cloned into pME3219 cut with *Eco*RI and *Pst*I. The resulting construct, pME3238, was thus equivalent to pME3225, except for an altered sequence of 21 bp between the FNR/ANR box and the -10 region. In the *hcn*-*lacZ* transcriptional fusion of pME6520, the *lacZ* gene with its own ribosome-binding site (from pME3535; Højberg *et al.*, 1999) was joined to a 380 bp *hcn* promoter fragment which had been amplified by primers 1 and 2 (Table 1) in vector pME6010. The fusion was performed between an artificial *Hind*III site created previously close to the *hcnA* translation start (Laville *et al.*, 1998) and a *Pst*I site at the 5' end of the *lacZ* fragment.

β -Galactosidase assay. β -Galactosidase specific activities were determined by the Miller method (Sambrook *et al.*, 1989).

RESULTS AND DISCUSSION

Anaerobic induction of the *hcnABC* genes depends on ANR and a specific ANR box

A translational *hcnA'*-*lacZ* fusion carried by pME3219 (Fig. 2) in strain CHA0 shows growth-phase-dependent expression, with highest β -galactosidase activities towards the end of growth in mildly oxygen-limited cultures (Blumer *et al.*, 1999). Both ANR and GacA contribute to this expression pattern (Blumer *et al.*, 1999). Here and in the following sections, *hcn* gene expression was mostly monitored at cell densities of about 10^9 cells ml^{-1} ($\text{OD}_{600} \cong 1.0$), at which both ANR and GacA can have significant effects, although the latter regulator has more impact at higher cell densities. The *hcn* promoter contains an FNR/ANR box (TTGGC...ATCAA) at about -40 nucleotides from the transcription start site (Fig. 1; Laville *et al.*, 1998). To prove that activation of this promoter by oxygen limitation is mediated by ANR at the ANR box, we constructed a series of *hcnA'*-*lacZ* fusion plasmids by deleting specific promoter sequences (Fig. 2). In plasmid pME3219 a 210 bp sequence lies upstream of the ANR box; this sequence was completely deleted in plasmid pME3225, leaving only the ANR box and the downstream promoter region (Fig. 2). In pME3223 the ANR box was removed, with only 35 bp of the promoter left

Fig. 2. Influence of strong oxygen limitation on the expression of *hcn* promoter deletion constructs. β -Galactosidase expression (Miller units) of translational *hcnA'*-*lacZ* fusions was tested in the *P. fluorescens* strains CHA0 (wild-type), CHA21 (*anr*) and CHA89 (*gacA*) when cells grown in NYB reached an OD₆₀₀ of 0.8–1.0. Activities are mean values of triplicate experiments $\pm$ SD. +O₂, aerobic growth conditions; -O₂, strongly oxygen-limiting growth conditions. The sequence of the stem-loop structure (Q) is shown in Fig. 3.

(Fig. 2). These constructs were introduced into strains CHA0 (wild-type) and CHA21 (*anr*), and recombinant strains were grown in NYB, either with good aeration or under severely oxygen-limiting (anaerobic) conditions to an OD₆₀₀ of 0.8–1.0. Strains CHA0/pME3219 and CHA0/pME3225 expressed similar, high β -galactosidase levels, which were inducible 40- to 60-fold by oxygen depletion (Fig. 2), indicating that the region located upstream of the ANR box is dispensable for induction by oxygen limitation. In the *anr* mutant CHA21, both constructs were expressed at very low levels (Fig. 2), reflecting an almost total requirement for the ANR protein. This strict ANR dependence is remarkable, considering the fact that the -40 region deviates in two positions from the consensus FNR/ANR recognition sequence (TTGAT . . . ATCAA). The construct lacking the ANR box, pME3223, showed drastically reduced β -galactosidase expression in anaerobiosis, whether or not ANR was present (Fig. 2). Thus, as postulated, the ANR box is responsible for anaerobic control by ANR. We also noted that in the absence of ANR all constructs tested yielded β -galactosidase values that were about twofold higher under anaerobic than under aerobic conditions. Such ANR-independent anaerobic regulation has also been observed in *P. aeruginosa* (Winteler & Haas, 1996).

The -10 region (TAGATT) was changed to a consensus σ^{70} -10 sequence (TATAAT) in the construct pME3226, which was otherwise identical to pME3219 (Fig. 2). This change of the -10 promoter element led to elevated aerobic expression, whereas anaerobic expression was reduced more than twofold. Thus, plasmid pME3226 showed a reduced ANR-dependent induction factor and higher expression levels in the *anr* mutant CHA21 (Fig. 2). In conclusion, the expression of

the native *hcn* promoter strongly depends on ANR and the ANR box.

The FNR consensus box at -40 (TTGAT . . . ATCAA) combined with the consensus σ^{70} -10 sequence (TATAAT) has previously been tested for induction by oxygen limitation in strain CHA0. When this artificial promoter was linked to the *lacZ* gene and integrated into the *P. fluorescens* chromosome, an anaerobic induction factor of about 25 was observed under batch conditions in flask cultures (Højberg *et al.*, 1999). When the same promoter construct was carried by the vector pME6010, which has about six copies per chromosome (Heeb *et al.*, 2000), the induction factor was the same (data not shown). Thus, the pME6010-based reporter plasmids used here are adequate to monitor ANR-dependent regulation.

The *hcn* promoter is not subject to GacA control

Although previous work (Blumer *et al.*, 1999) suggested that GacA control has a major impact on *hcn* gene expression at a post-transcriptional level, additional effects of GacA on the *hcn* promoter and a synergy with ANR were not excluded. Therefore, the same *hcnA'*-*lacZ* constructs were also tested in the *gacA* mutant CHA89 under aerobic and anaerobic conditions. All constructs that were anaerobically inducible in the wild-type strain CHA0 showed the same induction pattern in strain CHA89, but in general expression levels were about 20 times lower in the *gacA* mutant than in the wild-type (Fig. 2). All constructs used in Fig. 2 also gave strongly GacA-dependent expression in mildly oxygen-limited cultures grown in NYB to an OD₆₀₀ of 2.0–2.5 (data not shown). Thus, it is unlikely that GacA, or a regulator controlled by GacA, could activate the

Fig. 3. Influence of iron limitation on the expression of *hcn* promoter deletion constructs. β -Galactosidase expression (Miller units) of (a) translational *hcnA'*-*lacZ* fusions and (b) a transcriptional *hcn*-*lacZ* fusion was tested in the *P. fluorescens* strains CHA0 (wild-type), CHA21 (*anr*) and CHA89 (*gacA*) when mildly oxygen-limited cells reached an OD_{600} of 0.9–1.1. Activities are mean values of triplicate experiments $\pm$ SD. +Fe, growth in MMC supplemented with 20 μ M $FeCl_3$; -Fe, growth in MMC without $FeCl_3$; ND, not determined. Facing arrows indicate the inverted repeat forming a stem-loop structure (φ) in the 5' untranslated *hcn* leader sequence.

hcn promoter upstream of the ANR box or at the -10 hexamer. There was still the - somewhat remote - possibility that the *hcn* promoter sequence lying between the ANR box and -10 hexamer might have a role in GacA control. Therefore, a derivative of pME3225 (Fig. 2), pME3238, was constructed in which the *hcn* sequence (TATCCGTGTCCGACAAACCGA) was replaced by a random sequence (CGCTTACACTGGTAGCGTT-AG). However, this construct exhibited an unchanged dependence on GacA for expression (data not shown). Taken together, these results indicate that no element of the *hcn* promoter is involved in control by GacA. It appears that all effects of the GacS/GacA system on *hcnA* expression in *P. fluorescens* CHA0 occur post-transcriptionally, in agreement with earlier findings (Blumer *et al.*, 1999).

Iron regulation of *hcn* expression depends on ANR rather than on GacA

Under iron-limiting conditions, HCN production of *P. fluorescens* CHA0 is abolished (Voisard *et al.*, 1989).

Using again the translational *hcnA'*-*lacZ* fusion constructs, we examined the question of how iron limitation affects *hcn* expression in *P. fluorescens*. Strain CHA0 was grown in a minimal medium (MMC) with or without addition of 20 μ M $FeCl_3$. β -Galactosidase expression was determined when cells reached an OD_{600} of about 1.0. At this cell density, cultures grown without iron supplementation were in stationary phase and were green-fluorescent, which indicates siderophore (pyoverdine) production as a consequence of iron depletion, whereas cells grown in the presence of iron were in late-exponential-growth phase under mild oxygen limitation and did not produce siderophores. Iron depletion reduced the expression of the *lacZ* reporter fusion about 50-fold in strains CHA0/pME3219 and CHA0/pME3225 (Fig. 3a).

Many iron-regulated genes in bacteria are controlled by Fur (ferric uptake regulator), an aporepressor, which is converted to the active repressor by binding Fe^{2+} . Recent studies indicate that Fur could also act as an activator (Crosa, 1997). Therefore, as no *fur* mutant is available in

P. fluorescens CHA0, we tested the expression of pME3219 in the *fur* mutant PAO1A4 of *P. aeruginosa* (Barton *et al.*, 1996) and compared it with the expression in the wild-type strain PAO1 in NYB. No difference in expression was observed in both strains (data not shown), suggesting that iron regulation of the *P. fluorescens hcn* genes may be independent of Fur.

In eukaryotes, the expression of the iron storage protein ferritin is upregulated by an increase in the intracellular iron concentration. A stem-loop structure, the iron-response element, in the 5' leader of ferritin mRNA is involved in the regulation by iron (Adress *et al.*, 1997). The untranslated 5' leader of the *hcn* mRNA contains a predicted stem-loop structure (Laville *et al.*, 1998). By deleting a 17 bp *Ava*II fragment from the centre of the corresponding inverted repeat, plasmid pME3224 was created, the *hcn* mRNA of which could not form this specific secondary structure. The β -galactosidase activity of strain CHA0/pME3224, assayed under high and low iron conditions, was reduced, but still regulated by iron limitation (Fig. 3a), implying that the stem-loop structure may stabilize the transcript rather than affect the response to iron.

In the wild-type strain CHA0 carrying plasmid pME3223, which is deleted for the ANR box, β -galactosidase expression was no longer iron-dependent and in strain CHA0 harbouring pME3226, a construct which has lost most of its ANR-dependence because of an improved -10 consensus sequence (CTATAAT), β -galactosidase expression was constitutively high, in the presence and in the absence of iron (Fig. 3a). These results raised the possibility that the effects of iron limitation depend on ANR. Indeed, in the *anr* mutant CHA21 the expression of all constructs was no longer stimulated upon addition of iron (Fig. 3a).

In *E. coli*, iron-dependent transcriptional control can be mediated by FNR (Cotter *et al.*, 1992; Niehaus *et al.*, 1991). The transcriptionally active form of the FNR protein is a dimer containing two [4Fe-4S]²⁺ clusters. Oxygen inactivates FNR by converting the Fe-S cluster to a [2Fe-2S]²⁺ form (Kiley & Beinert, 1999). Iron depletion may limit the availability of Fe-S clusters to FNR and other Fe proteins (Cotter *et al.*, 1992). Since the cellular concentration of apo-FNR varies little under different growth conditions (Kiley & Beinert, 1999), FNR itself may act as an iron sensor, in addition to being an oxygen sensor (Rouault & Klausner, 1996). Considering the fact that FNR and ANR of *P. fluorescens* show a high degree of structural and functional conservation (Laville *et al.*, 1998; Højberg *et al.*, 1999), we suggest that *P. fluorescens* ANR could also sense iron availability and that iron depletion could convert ANR to an inactive state even under anaerobic conditions.

In the *anr* mutant CHA21, iron limitation and/or stationary-phase conditions caused somewhat elevated β -galactosidase expression levels of all reporter constructs (Fig. 3a), but in particular with pME3226. This effect may be explained by assuming that the house-keeping σ^{70} factor can recognize the *hcn* promoter,

especially that of pME3226, even without ANR. Moreover, in iron-deprived stationary-phase cells, the *hcn* promoter might be co-transcribed by RNA polymerase associated with the stress sigma factor σ^{38} (σ^S), which also recognizes the -10 sequence CTATAAT carried by pME3226 and which has no apparent need for a conserved -35 element, at least in *E. coli* (Wise *et al.*, 1996; Wösten, 1998). It has been shown previously that the σ^{70}/σ^{38} balance can affect the expression of secondary metabolism in *P. fluorescens* (Sarniguet *et al.*, 1995; Schnider *et al.*, 1995). Thus, in strain CHA0/pME3226, weak induction by ANR on the one hand and weak induction by iron depletion in stationary phase on the other hand might cancel each other out.

A transcriptional *hcn-lacZ* fusion in pME6520 escaped GacA control (Fig. 3b), as predicted, and was positively controlled by iron in *anr*⁺ strains, whereas the same fusion was upregulated slightly by iron depletion in the *anr* mutant CHA21 (Fig. 3b). These data reflect the fact that induction of *hcn* gene expression by high iron concentrations is essentially transcriptional and ANR-dependent. No evidence was obtained for iron effects at the post-transcriptional level, although the possibility that GacA might modulate siderophore synthesis and thereby indirectly influence the organism's response to iron availability cannot be excluded. Such an interpretation might explain why induction by iron sufficiency was less pronounced in a *gacA* background than in the wild-type (Fig. 3).

Conclusions

The *hcn* promoter of *P. fluorescens* CHA0 is anaerobically activated by ANR and this transcriptional activation by ANR appears to be mediated exclusively via the ANR box in the *hcn* promoter (Fig. 2). Moreover, *hcn* expression is strongly dependent on iron availability. Iron limitation has the same negative effect on *hcn* expression as has good aeration and this type of iron regulation is not observed in an *anr*-negative background (Figs 2 and 3). These results lead to the conclusion that ANR acts as an iron sensor, perhaps because iron limitation may restrict the assembly of the Fe-S clusters on the ANR apo-protein, a situation which would interfere with activation of ANR. As shown elsewhere (Blumer *et al.*, 1999), a region at or near the ribosome-binding site of the *hcnA* gene acts as a post-transcriptional target of the GacS/GacA regulatory network. The data obtained in this work suggest that ANR and the GacS/GacA system act sequentially, rather than synergistically, on cyanogenesis. Oxygen and iron availability determine the activity of ANR, whereas cell density appears to influence the activity of the GacS/GacA system, via unknown signals.

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