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. aeruginosa*. 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...
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; Schneider 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 (Reimmann et al., 1997; Zimmermann et al., 1991). GacA, the response regulator of the two-component regulatory system GacS (formerly LamA)/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; Ericksson 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 [4Fe–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; Höjberg et al., 1999). The transcription start site of the hcnABC gene cluster of P. fluorescens has been mapped (Fig. 1). The hcn promoter 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 arr 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) and CHA21 (arr::Km) (Laville et al., 1992, 1998). P. aeruginosa PA01 (wild-type) and PA01A4 (fur-4) (Barton et al., 1996) were used to assess iron regulation by Fur. Bacterial strains were routinely grown in nutrient yeast broth (Barton et al., 1996) or on nutrient agar plates (Stanisch & 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 μg ml⁻¹ (E. coli) or 125 μg 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 OD₅₆₀ 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 OD₅₆₀ of 0.8–1.0 (determined in a Pharmacia Ultrospec III spectrophotometer) corresponding to about 10⁷ 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
regulation of the hcn genes by iron. Cultures were grown in 50 ml flasks containing 20 ml MMC with shaking. An inoculum of ~4 x 10^5 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 wereverified 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 hcnA promoter region, using internal EcoRI-tagged primers which anneal to different sequences in the 5′ region of the hcnA 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 EcoRI and PstI and cloned into pME3219 previously cut with the same enzymes. The two-base substitution in the −10 region of the hcnA 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 AsrI deletion in the 5′ untranslated region of the hcnA genes (Fig. 3). For the construction of the deletion plasmid pME3238, a DNA linker with a 5′ EcoRI and a 3′ Mael site carrying the ANR box (underlined) and a random downstream sequence (bold face) not homologous to the native hcnA promoter sequence (5′-AATTCCGGCCAGATCAACGGCTTACATGTTAGCGTTAGC-3′) was synthesized. This linker was fused to a Mael/PstI fragment containing the authentic −10 region and the beginning of the hcnA gene (Laville et al., 1998) and cloned into pME3219 cut with EcoRI and PstI. 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 hcnA→lacZ transcriptional fusion of pME6520, the lacZ gene with its own ribosome-binding site (from pME3535; Højsberg et al., 1999) was joined to a 380 bp hcnA promoter fragment which had been amplified by primers 1 and 2 (Table 1) in vector pME6010. The fusion was performed between an artificial HindIII site created previously close to the hcnA translation start (Laville et al., 1998) and a PstI 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 CHAO 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 10^8 cells ml^-1 (OD_600 ± 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

### Table 1. Nucleotide sequences of the primers used for mutagenesis of the hcnA promoter region of P. fluorescens

<table>
<thead>
<tr>
<th>Primer</th>
<th>Resulting construct</th>
<th>Sequence*</th>
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<tr>
<td>Primer 3 (lacZ)</td>
<td>All†</td>
<td>5′-TGCTGCAAGGCGATTTAGG-3′</td>
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<td>5′-GCTGCAATCCATGCGTCATTTTCTT-3′</td>
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<tr>
<td>Primer 2</td>
<td>pME3226</td>
<td>5′-CATGGAACCTCATCCGTGAAAAATTAGTGA-3′</td>
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<td>Primer 7</td>
<td>pME3223</td>
<td>5′-GCTGCAATCCATCGTGTCGACAAACCAGC-3′</td>
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<td>Primer 8</td>
<td>pME3225</td>
<td>5′-GCTGCAATCTGTGGCCGATCAATATCC-3′</td>
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<tr>
<td>Primer 9</td>
<td>pME3226</td>
<td>5′-CAGACCTAATTGCGCCCGG-3′</td>
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* Additional bases at the 5′ end that are not homologous to the hcnA sequence are shown in bold face. Restriction sites (EcoRI, HindIII) 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.
The FNR consensus box at −40 (TTGAT...ATCA) combined with the consensus σ70−10 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 native hcn promoter strongly depends on ANR and the ANR box.

**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 ± SD. +O2, aerobic growth conditions; −O2, strongly oxygen-limiting growth conditions. The sequence of the stem–loop structure (9) is shown in Fig. 3.
**ANR-dependent iron control of cyanogenesis**

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**β-Galactosidase activity**

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**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. Activities are mean values of triplicate experiments ‡SD. +Fe, growth in MMC supplemented with 20 µM FeCl$_3$; fiFe, growth in MMC without FeCl$_3$; ND, not determined. Facing arrows indicate the inverted repeat forming a stem–loop structure (p) in the 5′ untranslated *hcn* leader sequence.

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**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 *hcn* 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 µM FeCl$_3$; −Fe, growth in MMC without FeCl$_3$; ND, not determined. Facing arrows indicate the inverted repeat forming a stem–loop structure (p) in the 5′ untranslated *hcn* leader sequence.

**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 PA01A4 of P. aeruginosa (Barton et al., 1996) and compared it with the expression in the wild-type strain PA01 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-responsive element, in the 5′ leader of ferritin mRNA is involved in the regulation by iron (Addess 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 AvaII 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; Högb erg 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 σ70 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 σs (σs), 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; Wosten, 1998). It has been shown previously that the σs/σs balance can affect the expression of secondary metabolism in P. fluorescens (Sarniguet et al., 1995; Schneider 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 anrr 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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