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Characterization of the *hcnABC* Gene Cluster Encoding Hydrogen Cyanide Synthase and Anaerobic Regulation by ANR in the Strictly Aerobic Biocontrol Agent *Pseudomonas fluorescens* CHA0

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The secondary metabolite hydrogen cyanide (HCN) is produced by *Pseudomonas fluorescens* from glycine, essentially under microaerophilic conditions. The genetic basis of HCN synthesis in *P. fluorescens* CHA0 was investigated. The contiguous structural genes *hcnABC* encoding HCN synthase were expressed from the T7 promoter in *Escherichia coli*, resulting in HCN production in this bacterium. Analysis of the nucleotide sequence of the *hcnABC* genes showed that each HCN synthase subunit was similar to known enzymes involved in hydrogen transfer, i.e., to formate dehydrogenase (for HcnA) or amino acid oxidases (for HcnB and HcnC). These similarities and the presence of flavin adenine dinucleotide- or NAD(P)-binding motifs in HcnB and HcnC suggest that HCN synthase may act as a dehydrogenase in the reaction leading from glycine to HCN and CO_2. The *hcnA* promoter was mapped by primer extension; the −40 sequence (TTGGC . . . .ATCAA) resembled the consensus FNR (fumarate and nitrate reductase regulator) binding sequence (TTGAT . . . .ATCAA). The gene encoding the FNR-like protein ANR (anaerobic regulator) was cloned from *P. fluorescens* CHA0 and sequenced. ANR of strain CHA0 was most similar to ANR of *P. aeruginosa* and CydR of *Azotobacter vinelandii*. An *anr* mutant of *P. fluorescens* (CHA21) produced little HCN and was unable to express an *hcnA-lacZ* translational fusion, whereas in wild-type strain CHA0, microaerophilic conditions strongly favored the expression of the *hcnA-lacZ* fusion. Mutant CHA21 as well as an *hcn* deletion mutant were impaired in their capacity to suppress black root rot of tobacco, a disease caused by *Thielaviopsis basicola*, under gnotobiotic conditions. This effect was most pronounced in water-saturated artificial soil, where the *anr* mutant had lost about 30% of disease suppression ability, compared with wild-type strain CHA0. These results show that the anaerobic regulator ANR is required for cyanide synthesis in the strictly aerobic strain CHA0 and suggest that ANR-mediated cyanogenesis contributes to the suppression of black root rot.

Cyanide is a secondary metabolite produced by some gram-negative bacteria, such as *Pseudomonas fluorescens*, *P. aeruginosa*, and *Chromobacterium violaceum* (1, 5, 26). Hydrogen cyanide (HCN) and CO_2 are formed stoichiometrically from glycine (6, 58) in a poorly understood oxidative reaction catalyzed by HCN synthase (4, 7). This enzyme or enzyme complex appears to be membrane bound (61). In extracts, HCN synthase of a *Pseudomonas* sp. oxidizes glycine in the presence of artificial electron acceptors, e.g., phenazine methosulfate (58). Flavin adenine dinucleotide (FAD) stimulates this reaction (59), whereas pyrrolnitrin, an inhibitor of many flavin enzymes, and *o*-phenanthroline, an iron chelator, strongly inhibit cyanide formation in vitro (58). HCN synthase is very sensitive to molecular oxygen and has been purified only partially from a *Pseudomonas* sp. and *P. aeruginosa* (4, 60). Nothing is known about the molecular structure of the enzyme.

In vivo, the four electrons produced by the HCN synthase reaction are transferred to oxygen, probably by components of the respiratory electron transport chain (4). In *P. aeruginosa*, no HCN is produced under fully anaerobic conditions when nitrate is the terminal electron acceptor (5). Optimal expression of HCN synthase occurs during the transition from the exponential to the stationary phase (9) and at low oxygen levels (8). Two regulatory proteins involved in these induction processes in *P. aeruginosa* have been identified: GacA and ANR (38, 66). The global activator GacA, a response regulator of a two-component system, positively controls the synthesis of HCN, other secondary metabolites, and exoenzymes by a cell-density-dependent mechanism (29, 38). The FNR-like anaerobic regulator ANR is required for the induction of HCN synthase, the arginine deiminase pathway, and the entire denitrification pathway (64, 66). *P. aeruginosa* mutants affected in either *gacA* or *anr* produce very little HCN (38, 66).

P. fluorescens CHA0 is an aerobic, root-colonizing biocontrol bacterium that protects several plants from root diseases caused by soilborne fungi (42, 52). HCN production by strain CHA0 contributes to the suppression of black root rot of tobacco, a disease caused by *Thielaviopsis basicola*, under gnotobiotic conditions (53). GacA-negative mutants of strain CHA0, which are pleiotropically defective in the synthesis of HCN, antibiotics, and exoenzymes, have lost the ability to protect tobacco from black root rot (29, 39). We previously isolated HCN biosynthetic genes from strain CHA0 and demonstrated their expression in other pseudomonads, with a concomitant improvement in biocontrol ability (15, 53). When the

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype	Reference or source
Strains		
P. fluorescens		
CHA0	Wild type	52
CHA5	*hcnB*::Ω-Hg	53
CHA21	*anr*::Ω-Km	This study (Fig. 4)
CHA77	Δ*hcnABC*	This study (Fig. 1)
P3	Wild type	53
P. aeruginosa		
PAO1	Wild type	ATCC 15692
ADD1976	Chromosomal insertion of T7 *pol lacI*^{q}	2
E. coli		
BL21(DE3)	*hsdS gal*; chromosomal insertion of T7 *pol*	44
DH5α	*recA1 endA1 hsdR17 supE4 gyrA96 relA1* Δ(*lacZYA-argF*)*U169* (ϕ80d*lacZ*Δ*M15*)	40
JRG1728	Δ(*lacZYA*)*X74 galU galK* Δ(*ara-leu*) *rpsL* Δ(*tyrR-fnr-trg*)	17; J. R. Guest
RU4420	*thi-1 endA1 hsdR17 supE44 trp*::Tn*1725*	47
Plasmids		
pBluescript II KS$^+$	Cloning vector; ColE1 replicon; Ap^r	Stratagene
pEB16	Broad-host-range vector; pBR322-pRO1600 replicon; RK2 Mob; Ap^r Cb^r	2
pHP45Ω-Km	ColE1 replicon; Ap^r Km^r	14
pMMB67EH	RSF1010 replicon; Ap^r	16
pNM482	ColE1 replicon; Ap^r *'lacZ*	34
pVK100	Broad-host-range vector; IncP replicon; RK2 Mob; Tc^r Km^r	25
pME497	Mobilizing plasmid; IncP replicon; Tra; Ap^r	54
pME3013	pVK100 with an 8-kb *Hin*dIII genomic fragment of *P. fluorescens* CHA0 containing the *hcnABC* genes for HCN biosynthesis	53
pME3071	pMMB67 with a 5-kb *Xho*I-*Hin*dIII genomic fragment of CHA0 containing *hcnABC′*	This study (Fig. 1)
pME3087	Suicide vector; ColE1 replicon; RK2 Mob; Tc^r	52
pME3205	pVK100 with a 3.8-kb *Sal*I-*Hin*dIII fragment of pME3071 containing the *hcnABC′* genes behind the kanamycin promoter	This study (Fig. 1)
pME3206	pVK100 with a 3.6-kb *Mae*I-*Hin*dIII fragment of pME3071 containing the *hcnABC′* genes behind the kanamycin promoter	This study (Fig. 1)
pME3209	pEB16 with a 1.3-kb *Bam*HI-*Hin*dIII fragment of pME3071 containing the *hcnC′* gene behind the T7 promoter	This study (Fig. 1)
pME3210	pEB16 with a 3.6-kb *Mae*I-*Hin*dIII fragment of pME3071 containing the *hcnABC′* genes behind the T7 promoter	This study (Fig. 1)
pME3212	pEB16 with a 3.65-kb *Mae*I-*Stu*I fragment of pME3071 containing the *hcnABC* genes behind the T7 promoter	This study (Fig. 1)
pME3219	pME6010 with a 410-bp *hcnA* promoter fragment and an *hcnA′-'lacZ* translational fusion at the *Pst*I site in *hcnA*	This study
pME3580	pKT240 with a 1.3-kb *Sac*II genomic fragment of *P. aeruginosa* PAO1 containing the *anr* gene	17
pME3812	pVK100 with a 19-kb *Hin*dIII genomic fragment of *P. fluorescens* CHA0 containing the *anr* gene	This study
pME3815	pBluescript II KS$^+$ with a 2.5-kb *Pst*I fragment of pME3812 containing the *anr* gene	This study (Fig. 4)
pME3816	pME3087 Δ*Eco*RI[a] with a 4.7-kb fragment containing *anr*::Ω-Km	This study (Fig. 4)
pME3817	pVK100 with a 1.6-kb *Bam*HI-*Hin*dIII fragment of pME3818	This study (Fig. 4)
pME3818	pME3815 with a deletion of the 0.9-kb *Eco*RV fragment	This study (Fig. 4)
pME6010	pACYC177-pVS1 shuttle vector; Tc^r	S. Heeb (20a)

[a] Δ*Eco*RI signifies that the *Eco*RI site of pME3087 was deleted.

hcn structural genes are inactivated by insertion of a resistance cassette, strain CHA0 loses part of its ability to suppress black root rot. This defect can be restored by complementation with a plasmid carrying the *hcn* genes (53). Here we show that the *hcn* genes are organized as an *hcnABC* cluster which appears to be sufficient to encode HCN synthase. We also characterize the *P. fluorescens anr* gene, whose function is essential for the expression of the *hcnABC* cluster at low oxygen concentrations. Finally, we assess the importance of *anr*-dependent regulation for biocontrol by strain CHA0.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids are listed in Table 1. Strains of *Escherichia coli* and *P. aeruginosa* were routinely grown on nutrient agar (NA) plates and in nutrient yeast broth (NYB) with aeration at 37°C (18). Anaerobic growth and gas production of *E. coli* were assessed as described previously (17). For determination of HCN production by *E. coli*, strains were cultivated in a medium [LB(2x)-M9-MMC] containing, per liter, the following: tryptone (Oxoid), 20 g; yeast extract, 10 g; glucose, 4 g; glycine, 0.75 g; L-methionine, 1.5 g; NaCl, 2.5 g; NH_4Cl, 1 g; KH_2PO_4, 3 g; Na_2HPO_4, 6 g; and $FeCl_3$, 0.5 g. Minimal medium M9 (40) supplemented with 0.5% Methionine Assay Medium (Difco) was used for protein expression in *P. aeruginosa* ADD1976. *P. fluorescens* cells were routinely cultivated in NYB or on NA at 30°C. To measure HCN production by *P. fluorescens*, strains were grown under oxygen-limited conditions in tightly closed 120-ml bottles containing a synthetic minimal medium (MMC) described by Castric (5). In *hcnA′-'lacZ* expression experiments, MMC was also used in Erlenmeyer flasks, with shaking (180 rpm) to provide good aeration. For the determination of arginine deiminase activity and for the experiment measuring competition between strains CHA0 and CHA21, yeast extract-arginine (YEA) medium (49) was used. Antimicrobial compounds, when required, were added to the growth media at the following

concentrations: ampicillin, 100 μg/ml (for *E. coli*); carbenicillin, 200 μg/ml (for *E. coli*); kanamycin sulfate, 25 μg/ml; $HgCl_2$, 20 μg/ml; and tetracycline hydrochloride, 25 μg/ml (for *E. coli*) or 125 μg/ml (for *P. fluorescens*). 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) was incorporated into solid media to monitor β-galactosidase expression (40).

DNA manipulations and sequencing. Small-scale preparations of plasmid DNA from *E. coli* and *P. fluorescens* were carried out by the CTAB method (12) for ColE1-based plasmids and pMMB67 or by the alkaline lysis method (40) for other plasmids. Large-scale preparations of plasmid DNA were carried out with Qiagen Tips (Qiagen Inc.). Restriction enzyme digestions, DNA fragment isolation from low-melting-point agarose gels, ligation, and agarose gel electrophoresis were performed according to standard procedures (40). Chromosomal DNA of *P. fluorescens* was isolated as described by Gamper et al. (18). Transformation of *E. coli* and *P. aeruginosa* strains with plasmid DNA was done by the standard $CaCl_2$ procedure (40). Progressive deletions with nuclease *Bal* 31 were performed according to the instructions of the supplier (Boehringer Mannheim Biochemicals). For nucleotide sequence determination of the *hcn* genes, DNA fragments were cloned into M13mp18 and M13mp19 phages (63), and single-stranded DNA was sequenced by the dideoxy chain termination method with [α-^{32}P]dATP, 7-deaza-dGTP, and Sequenase version 2.0 (United States Biochemical Corp.). PCR was carried out with plasmid DNA (0.5 μg) carrying the *hcn* genes as the template. Two oligonucleotide primers were used. Primer 1 anneals to ORF0 upstream of the *hcn* promoter (5′-GCTCGAATTCCTGCGT CATTACTCTT-3′) and contains an *Eco*RI restriction site (underlined) at the 5′ end. Primer 2 anneals to the ribosome-binding site upstream of the ATG at position 238 (see Fig. 2) (5′-CATGCAAGCTTCATCCGTGAAAAATGAAT G-3′) and contains a *Hin*dIII restriction site (underlined) at the 5′ end. For the amplification reactions, the thermostable DNA polymerase PRIME ZYME (Biometra) was used. Thermal cycling (12 cycles) consisted of denaturation at 95°C for 1 min, primer annealing at 56°C for 1 min, and elongation at 72°C for 1.5 min. The PCR fragment obtained with primers 1 and 2 contained an artificial *Hin*dIII site, to which a *′lacZ* fragment was fused as previously described (34). The unique *Pst*I site in the *hcnA* gene was used to create a second *′lacZ* translational fusion. The vector for these fusions was pME6010, a pACYC177-pVS1 shuttle vector (20a).

The *anr* nucleotide sequence was determined by Genome Express (Grenoble, France). Nucleotide and deduced amino acid sequences were analyzed with the programs GAP (whole-length sequence alignments), PILEUP (multiple alignments), and BLAST by use of the Genetics Computer Group package (version 8).

Bacterial matings. Triparental matings of *P. fluorescens* recipients with *E. coli* containing a mobilizable plasmid (pVK100 or pME3087) and with *E. coli* containing a mobilizing plasmid (pME497) were performed as previously described (52).

Tn*1725* mutagenesis. *E. coli* RU4420 (47) harboring pME3013 was spread on NA plates containing a chloramphenicol (0 to 1,000 μg/ml) gradient. Highly resistant colonies were purified on NA supplemented with chloramphenicol (500 μg/ml). They carried pME3013::Tn*1725* derivatives.

Construction of *P. fluorescens* mutants by gene replacement. For construction of the chromosomal *hcn* deletion mutant CHA77 (Fig. 1), a 2.4-kb *Pst*I deletion was created within the *hcn* genes of pME3071. The *hcn* flanking sequences were cloned into the suicide vector pME3087, which carries a tetracycline resistance determinant (52). To obtain strain CHA21, in which the chromosomal *anr* gene is disrupted by the Ω-Km element (see Fig. 4), a chromosomal *Pst*I fragment of pME3815 containing the *anr* gene was cloned into vector pME3087 with a deletion of its *Eco*RI site. The Ω-Km fragment of pHP45Ω was inserted into the unique *Eco*RI site within the *anr* gene. Suicide plasmids carrying either the *hcn* gene deletion or the *anr* gene disruption were mobilized by helper plasmid pME497 to wild-type strain CHA0 and chromosomally integrated, with selection for tetracycline resistance. Excision of the vector by a second crossover was carried out by enrichment for tetracycline-sensitive cells (64). Selection for kanamycin resistance ensured the presence of the Ω-Km insertion in strain CHA21. Both mutations were checked by Southern blotting (data not shown) and by testing of their HCN-negative phenotype.

Primer extension analysis. Extraction of total RNA from *P. fluorescens* was performed according to the method reported by Kullik et al. (27). Primer extension reactions were carried out essentially as described previously (51). The oligonucleotides used as primers for cDNA synthesis, 5′-GGGGTTGCCGGCG TCCCGCCGTCCATGCTGC-3′ (HCN4; positions 218 to 188) and 5′-GCTGC TGCGGTCGGGACCGGGCAACGTCC-3′ (HCN5; positions 192 to 164), both annealed to the coding strand of *hcnA* (Fig. 2). The oligonucleotides (5 to 10 pmol each) were 5′ labeled with 10 U of T4 polynucleotide kinase (Pharmacia) and 20 μCi of [α-^{32}P]dATP at 37°C for 30 min. Nonincorporated nucleotides were eliminated by passage over a Sephadex G-25 column. The primer elongation reaction was carried out at 43°C for 1 h with reverse transcriptase SuperScript (GIBCO BRL) and 7-deaza-dGTP. Unlabeled primers were used to generate a nucleotide sequence ladder upstream of the *hcnA* gene. Primer extension products were run in parallel with the sequencing reaction to map the transcription initiation site.

Colony hybridization. A genomic library of *P. fluorescens* CHA0 cloned into pVK100 (53) was screened in *E. coli* with a probe containing most of the *anr* gene of *P. aeruginosa* (see Fig. 4). A 0.6-kb *Eco*RI-*Apa*I fragment of pME3580 (Table 1; see Fig. 4), which served as the probe, was excised from a low-melting-point agarose gel and labeled with [α-^{32}P]dATP by the random-primer method (13). Colony hybridization with Hybond-N membranes was performed according to the protocol of the supplier (Amersham) and Perbal (37). Prehybridization, hybridization, and washing were carried out at 65°C (high-stringency conditions).

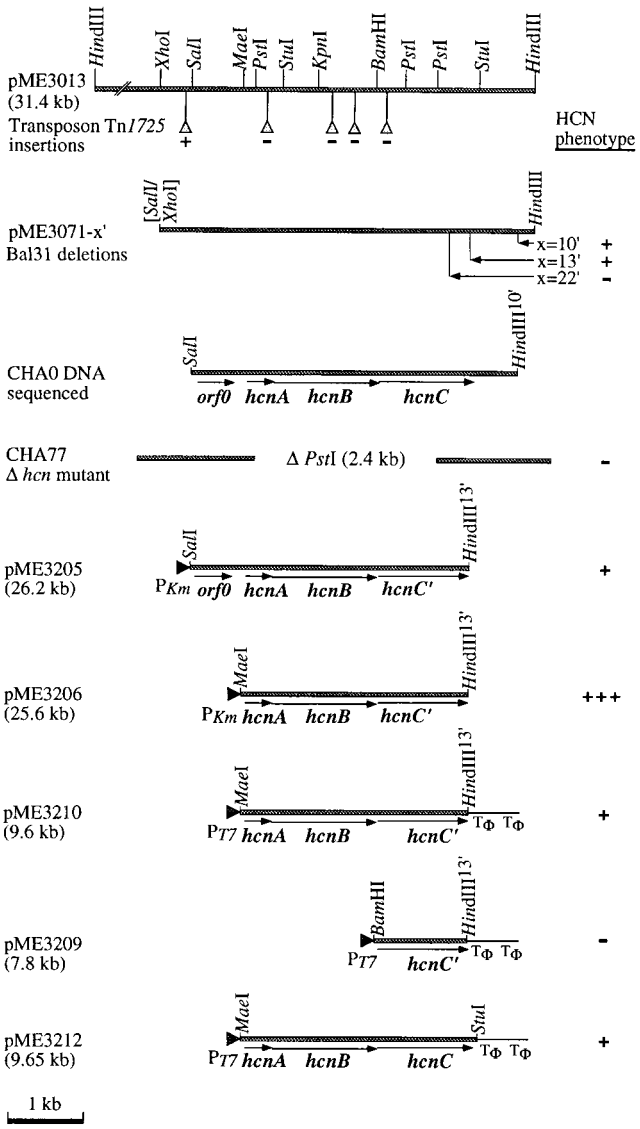

FIG. 1. Recombinant plasmids carrying the *hcnABC* region from *P. fluorescens* CHA0. Symbols: ▭, genomic DNA from strain CHA0; △, sites of Tn*1725* insertions; ▶, orientation of kanamycin resistance and T7 promoters (P_{Km} and P_{T7}, respectively) on vector plasmids; T_Φ, transcription terminator; x, time of *Bal* 31 digestion (minutes) and extent of deletions created by *Bal* 31. The HCN phenotype was assessed by qualitative and quantitative tests for HCN production as follows: −, <5 μM HCN; +, wild-type levels of HCN; overproduction of HCN.

Protein expression. The *hcn* genes of *P. fluorescens* were cloned under the control of the T7 promoter in pEB16, producing pME3209, pME3210, and pME3212 (Table 1 and Fig. 1). NYB cultures of *P. aeruginosa* ADD1976 (2) harboring any of these constructs were grown at 37°C until they reached an optical density at 600 nm of 0.8. Cells were centrifuged and resuspended in minimal medium M9 (40) supplemented with 0.5% Methionine Assay Medium, which contains all essential growth factors except methionine. After incubation at 37°C with shaking for 75 min, the chromosomal T7 RNA polymerase was induced by the addition of 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 1 h. Rifampin was added at 200 μg/ml and, after further incubation for 30 min, 10 μCi of L-[^{35}S]methionine (Amersham) was mixed with the culture. After 50 min at 37°C, cells were harvested, washed in 50 mM glucose solution buffered with Tris-HCl (25 mM, pH 8.0), and lysed for 5 min at 100°C in sample buffer containing 62.5 mM Tris-HCl (pH 8.8), 2% (wt/vol) sodium dodecyl sulfate

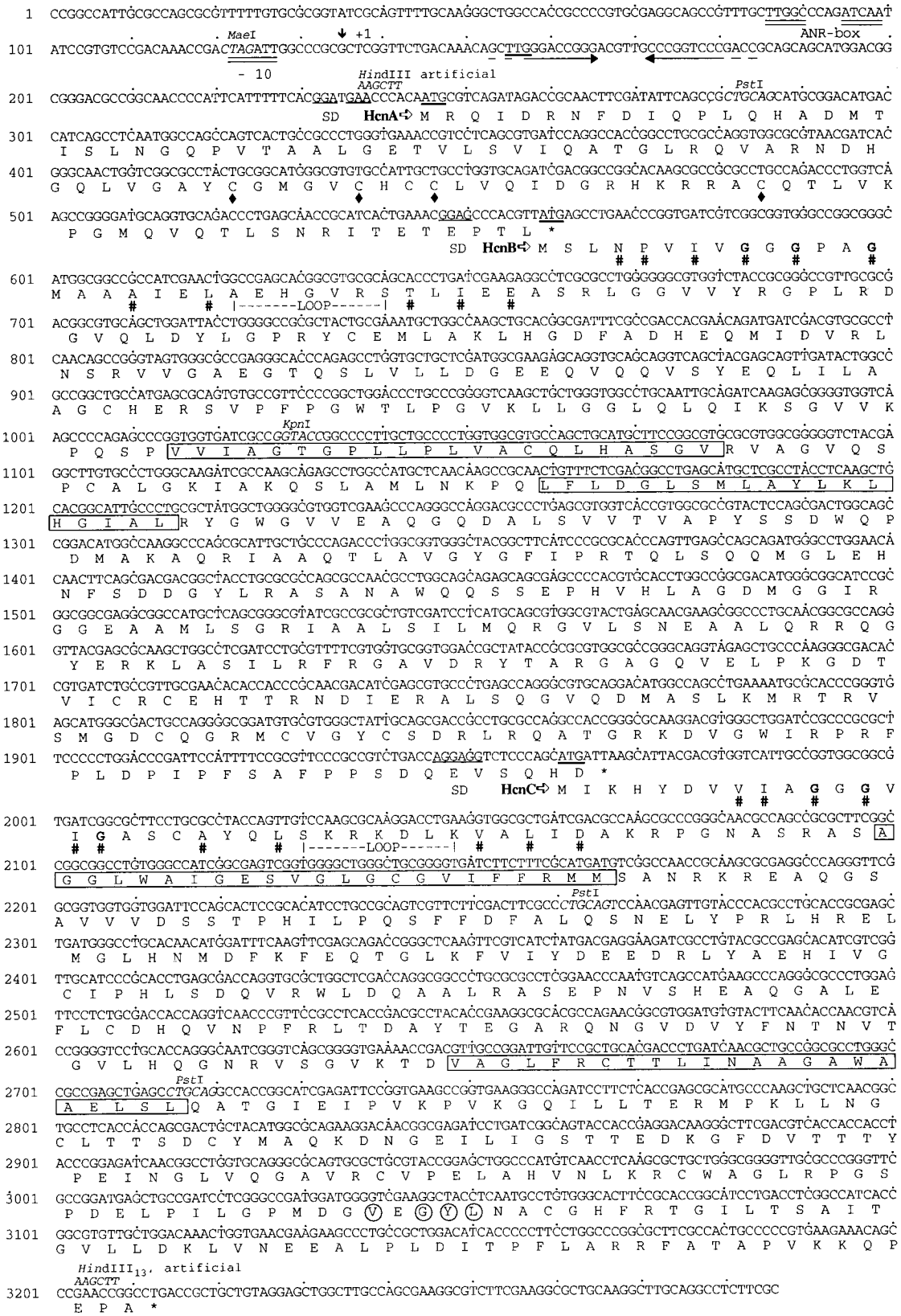

FIG. 2. Nucleotide sequence of the *hcn* gene cluster of *P. fluorescens* CHA0 and deduced amino acid sequence of its protein products. The putative start codons and potential ribosome-binding site (SD) are underlined. The transcription start site is marked with +1. Double underlining below the sequence indicates a sequence (ANR-box) homologous to the *E. coli* FNR-binding site and the −10 region of the *hcn* promoter. Facing arrows indicate inverted repeats. Four cysteine residues in HcnA discussed as potential binding sites for an Fe—S cluster are indicated by diamonds. Eleven amino acid residues within HcnB and HcnC that define an ADP-binding motif, including three conserved glycine residues (boldface) (56), are marked by number signs, and the variable loop is indicated. Predicted transmembrane segments in HcnB and HcnC are boxed. Four amino acid residues in the C-terminal region of HcnC which are identical to the *E. coli* Pfl region containing the free glycyl radical in activated Pfl are circled. Introduction of artificial *Hin*dIII sites at the 5′ end of the *hcnA* gene and at the 3′ end of the truncated *hcnC′* gene is described in the text.

(SDS), 10% (vol/vol) glycerol, 5% (vol/vol) β-mercaptoethanol, and 0.005% bromophenol blue. Aliquots were electrophoresed on SDS–15% (wt/vol) polyacrylamide gels (28). Dried gels were autoradiographed with a screen at −80°C for 6 to 12 h.

Production of secondary metabolites. HCN was quantified in *P. fluorescens* culture supernatants as described previously (19, 53). *E. coli* cultures were grown in closed 20-ml flasks containing 8 ml of LB(2x)-M9-MMC medium at 37°C with shaking. In this medium, HCN production by *E. coli* was high and cells were lysed at the end of growth, even when the T7 promoter had not been induced by IPTG. Since the expression of the *hcn* genes was toxic for *E. coli*, experiments were performed with freshly transformed cells, and carbenicillin (200 μg/ml) was used instead of ampicillin to prevent the loss of the *hcn* plasmid. After 24 h of incubation, HCN concentrations in the culture supernatants were determined by the method of Gewitz et al. (19). Strains growing on plates were tested qualitatively for HCN production by the indicator paper method (3). The antibiotics 2,4-diacetylphloroglucinol and pyoluteorin were quantified by established procedures (23).

Enzyme assays. Arginine deiminase was measured in toluene-treated cells (32). β-Galactosidase specific activities were determined by the Miller method (40).

Gnotobiotic system. Suppression of black root rot caused by *T. basicola* was determined with gnotobiotically grown tobacco plants as previously described (23, 53). Soil water content and soil water potential in the artificial soil of the gnotobiotic system were determined at the beginning of the experiments according to McInnes et al. (33).

Nucleotide sequence accession numbers. The nucleotide sequences of the *hcnABC* and the *anr* genes reported here have been assigned GenBank accession numbers AF053760 and AF053611, respectively.

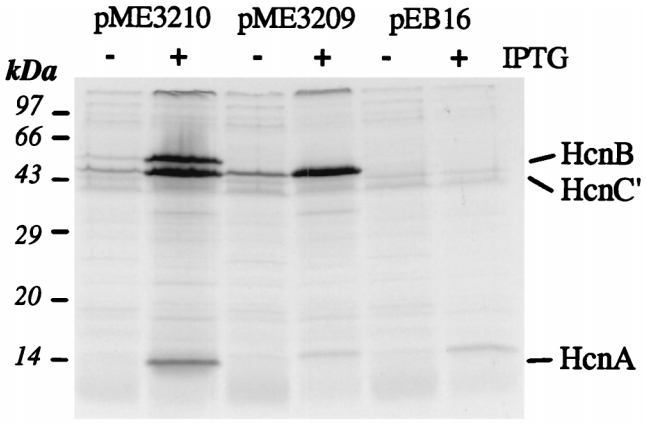

FIG. 3. Expression of the proteins HcnA, HcnB, and HcnC′. The *hcnABC′* genes were cloned in the T7 expression vector pEB16, giving plasmids pME3210 (*hcnABC′*) and pME3209 (*hcnC′*) (Fig. 1). Cultures of *P. aeruginosa* ADD1976 carrying either of these plasmids were induced with IPTG (2 mM) (+) or not induced (−). The proteins were labeled with [^{35}S]methionine, separated by electrophoresis in an SDS–15% polyacrylamide gel, and visualized by autoradiography.

RESULTS

Nucleotide sequence analysis of the *hcn* genes. A cyanide biosynthetic locus (*hcn*) of *P. fluorescens* CHA0 is carried by recombinant plasmid pME3013 (Fig. 1), previously described (53). To localize the *hcn* genes, we isolated Tn*1725* insertions in pME3013 and generated progressive deletions with *Bal* 31 in a derivative of pME3013, pME3071 (Fig. 1). These constructs were mobilized into *P. fluorescens* P3, a strain that does not produce HCN naturally but will do so when carrying pME3013 (53). In this way, the HCN biosynthetic capacity was localized to a 3.8-kb fragment, which was inserted into the broad-host-range vector pVK100, giving pME3205 (Fig. 1).

The nucleotide sequence of the 3.8-kb fragment was determined. Three contiguous open reading frames (ORFs), designated *hcnABC*, and the 3′ region of an additional ORF (ORF0) were found. ORF0 could be deleted in pME3206 (Fig. 1) without loss of cyanogenic capacity and was not analyzed further. The entire *hcnABC* region contained 66.2% G+C. The codon usage and the high G+C content at the third codon position (*hcnA*, 84%; *hcnB*, 89%; *hcnC*, 89%) were typical of *Pseudomonas* genes. The first ORF, *hcnA*, has two potential start codons, the ATG at position 245 and the upstream TTG at position 155 (Fig. 2). In order to determine the in vivo translation start site, we constructed two *′lacZ* translational fusions, one at an artificial *Hin*dIII restriction site created by PCR at position 237 and the other at the natural *Pst*I site located at position 282 (Fig. 2). In *P. fluorescens* CHA0, only the downstream *hcnA′-′lacZ* fusion, generated at the *Pst*I site, gave measurable β-galactosidase activity (data not shown), indicating that in vivo translation of *hcnA* starts at the ATG codon. The molecular mass of the deduced HcnA polypeptide is 11,525 Da. The most probable ATG start codon of the second ORF, *hcnB*, overlaps the TGA stop codon of *hcnA* (Fig. 2). The expression of this ORF was verified with a *lacZ* translational fusion constructed at the unique *Kpn*I site (Fig. 2) within *hcnB* (data not shown). The nucleotide sequence predicted a polypeptide of 50,647 Da for HcnB. For the third ORF, *hcnC*, the most likely ATG start codon is 2 bp upstream of the TAA stop codon of *hcnB* (Fig. 2). In the *Bal* 31-generated construct pME3071-10′, which gives an Hcn$^+$ phenotype in strain P3, the distance from the TGA stop codon of *hcnC* to the 3′ end of the insert (marked by a *Hin*dIII linker) is 0.7 kb (Fig. 1). In the subsequent deletion construct pME3071-13′, the last three codons and the stop signal of *hcnC* were removed (Fig. 2) without affecting the Hcn$^+$ phenotype conferred by this plasmid (Fig. 1). The deduced full-length HcnC polypeptide has a calculated molecular mass of 45,334 Da. The *hcnABC′* segment derived from pME3071-13′ was inserted behind the T7 promoter in pME3210 (Fig. 1). In this context, the truncated HcnC protein (HcnC′) had a tail of a short peptide (KLGASRGSGS) resulting from the fusion of the truncated *hcnC′* gene to the transcription terminator sequence of the vector. Thus, HcnC′ consists of a 46-kDa polypeptide. When the insert of pME3210 was expressed by T7 RNA polymerase in *P. aeruginosa* ADD1976, three polypeptides of approximately 12, 45, and 50 kDa were produced, corresponding to HcnA, HcnC′, and HcnB, respectively (Fig. 3). The *hcnC′* construct pME3209 (Fig. 1) expressed in strain ADD1976 produced the expected 45-kDa band only (Fig. 3). Thus, the *hcnABC* gene products seen were in agreement with the nucleotide sequence data.

The *hcnABC* cluster encodes HCN synthase. A 2.4-kb *Pst*I deletion was created within the *hcnABC* genes and transferred to the chromosome of strain CHA0 by a double-crossover technique previously described (64). The Δ*hcn* mutant CHA77 obtained did not produce measurable amounts of HCN, whereas the wild-type strain CHA0 and the complemented mutant CHA77/pME3013 did (Table 2). In *E. coli*, a bacterium that does not produce HCN naturally, the T7 expression construct pME3210 (*hcnABC′*) led to HCN production (Table 2) in the presence of low levels of T7 RNA polymerase (see Materials and Methods). The induction of T7 RNA polymerase was avoided to prevent host cell death by an HCN overdose. The T7 expression construct pME3212, containing the full-length *hcnABC* cluster (derived from pME3071-10′; Fig. 1), produced a similar amount of HCN (Table 2), indicating that the three C-terminal amino acid residues of HcnC (which are missing in HcnC′ carried by pME3210) are not essential for HCN synthase activity. Taken together, these results indicate that the *hcnABC* genes are the structural genes for HCN synthase. Their structural organization suggests that they form an operon.

TABLE 2. HCN biosynthesis in *P. fluorescens* CHA0 and *E. coli* BL21 harboring *hcnABC* recombinant plasmids

Strain	Relevant genotype	HCN production[a] (μM)
CHA0	*hcnABC*$^+$	89 ± 5
CHA77	Δ*hcn*	<5
CHA77/pME3013	*hcnABC*$^+$	96 ± 32
CHA77/pME3206	P_{Km}-*hcnABC*$^+$	855 ± 90
BL21/pEB16		<5
BL21/pME3210	P_{T7}-*hcnABC′*	132 ± 25
BL21/pME3212	P_{T7}-*hcnABC*$^+$	102 ± 13

[a] *P. fluorescens* was grown in MMC with O_2 limitation and *E. coli* was grown in a rich, glucose-containing medium as indicated in Materials and Methods. Mean ± standard deviation values for triplicate experiments are given.

Similarities between the HcnABC polypeptides and dehydrogenases. At the amino acid sequence level, HcnA has 34% identity with the α subunit of formate dehydrogenase from *Moorella thermoacetica* (*Clostridium thermoaceticum*; GenBank accession no. U73807). HcnA also has 31% identity with the HoxU subunit of hydrogenase from *Anabaena variabilis* (GenBank accession no. X79285) (41). A cluster of cysteine residues (Cys-X_4-Cys-X_2-Cys-X_n-Cys) in HcnA (Fig. 2) resembles a similar sequence motif in ferredoxins and may interact with a [2Fe—2S] center (46). HcnB and HcnC each have a typical FAD- or NAD(P)-binding motif, the ADP-binding βαβ fold (56), in their N-terminal parts (Fig. 2). HcnB is most similar to the SoxA subunit of sarcosine (*N*-methylglycine) oxidase from *Corynebacterium* sp. (32% identity in a stretch of 171 amino acid residues; GenBank accession no. Q46337) (10), to the OoxA subunit of octopine oxidase from *Agrobacterium tumefaciens* (30% identity; GenBank accession no. Z30328), and to the NoxA subunit of nopaline oxidase from the same organism (30% identity; GenBank accession no. Z30316) (65). HcnC is most similar to the DadA subunit of a putative D-amino acid oxidase from *P. aeruginosa* (31% identity; GenBank accession no. L48934) and to the homologous protein from *E. coli* (23% identity; GenBank accession no. P29011) (31). The SoxB subunit of sarcosine oxidase from *Corynebacterium* sp. (GenBank accession no. P40875) and HcnC also resemble each other (24% identity). The significance of these similarities is considered in the Discussion.

Mapping of the *hcnA* promoter. RNA preparations from CHA0 cultures harvested at various growth phases were used for mapping the 5′ end of the *hcnA* transcript by primer extension (data not shown). The +1 site determined reveals a −10 sequence (TAGATT) and an FNR/ANR box which is centered around −41.5 (TTGGC. . . .ATCAA; Fig. 2) and which deviates in two positions from the consensus FNR recognition sequence (TTGAT. . . .ATCAA) (43, 57). The −41.5 location of an FNR/ANR box is typical of anaerobically inducible promoters that are controlled by FNR or FNR-like regulators (43). Since strain CHA0, like other pseudomonads, produces HCN optimally in oxygen-limited cultures (8, 53), the existence of an appropriately positioned FNR/ANR box in the *hcnA* promoter suggested that strain CHA0 may have an *anr* gene, which could positively control HCN production.

Cloning of the *P. fluorescens anr* gene. An *Eco*RI-*Apa*I fragment carrying most of the *P. aeruginosa anr* gene (66) was used as a probe to screen a genomic library of *P. fluorescens* CHA0 established in cosmid pVK100 in *E. coli* (53). Three clones detected by colony hybridization each carried a pVK100 derivative with a common 19-kb *Hin*dIII insert. One recombinant cosmid, pME3812, was retained. It contained an internal

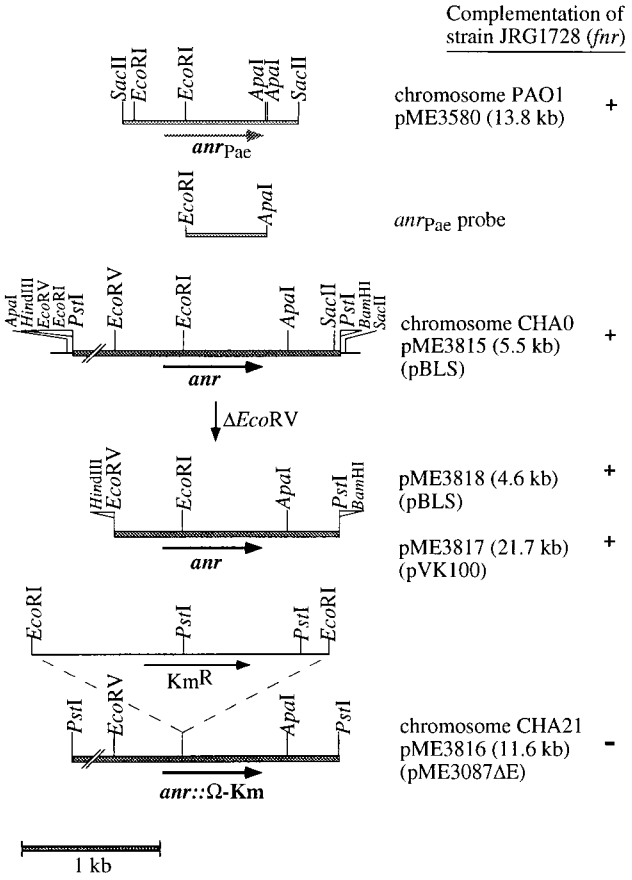

FIG. 4. Cloning strategy for the *anr* gene of *P. fluorescens* CHA0 and mutant construction. Details are explained in the text. Plasmids are listed in Table 1. +, positive for complementation of the *E. coli fnr* mutant JRG1728; −, negative for complementation; anr_{Pae}, *anr* gene of *P. aeruginosa.*

2.5-kb *Pst*I fragment hybridizing to the *anr* gene of *P. aeruginosa*; the *P. fluorescens* origin of the 2.5-kb fragment was confirmed by Southern hybridization of *P. fluorescens* genomic DNA digested with *Pst*I (data not shown). The 2.5-kb fragment was subcloned into pBluescript II KS$^+$ resulting in pME3815. After removal of a 0.9-kb upstream segment by *Eco*RV digestion, pME3818 was obtained (Fig. 4). The *E. coli fnr* mutant JRG1728 was complemented for anaerobic gas production and anaerobic growth on glycerol-nitrate medium by plasmids pME3812, pME3815, and pME3818, indicating that they all contained a functional *fnr* homolog (Fig. 4). The *anr* gene of *P. fluorescens* was subcloned into broad-host-range vector pVK100. The recombinant plasmid obtained, pME3817, also complemented the *fnr* mutant of *E. coli* (Fig. 4). In these assays, pME3580 carrying the *anr* gene of *P. aeruginosa* (17) was included as a positive control.

The nucleotide sequence of the 1.6-kb insert of pME3818 revealed the *anr* gene of *P. fluorescens* CHA0. Its deduced product consists of 244 amino acid residues and has a calculated molecular mass of 27,155 Da. The G+C content of the *anr* gene is 61.5%.

Similarity among ANR of *P. fluorescens*, ANR of *P. aeruginosa*, and FNR of *E. coli*. The deduced amino acid sequence of ANR of *P. fluorescens* (ANR_{Pfl}) shows overall identities of 88% (95% similarity) with the sequence of *P. aeruginosa* ANR (ANR_{Pae}) and of 53% (76% similarity) with the sequence of *E. coli* FNR (Fig. 5). In FNR, three N-terminal cysteine residues

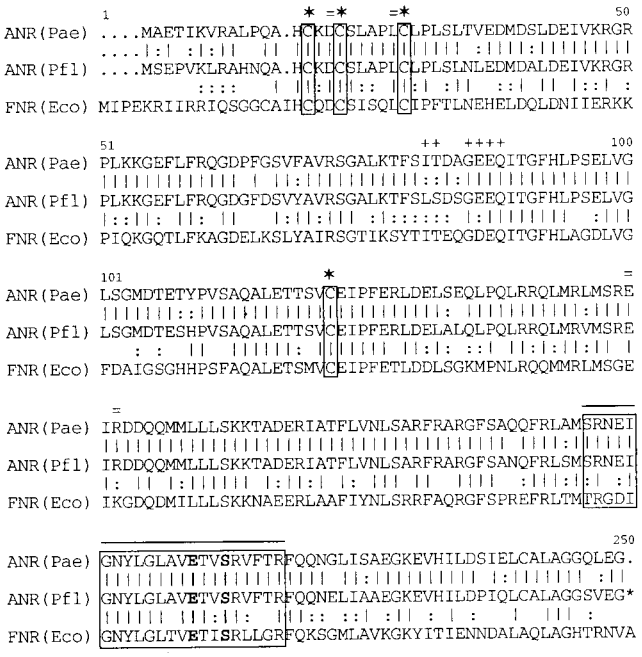

FIG. 5. Alignment of the deduced amino acid sequence of ANR from *P. fluorescens* (Pfl) with those of *P. aeruginosa* (Pae) ANR and *E. coli* (Eco) FNR. The sequences were aligned by use of the computer programs PILEUP and GAP. Identities are indicated by vertical lines, and similarities are indicated by colons. The following features of the *E. coli* FNR protein are highlighted: ★, cysteine residues required for activity; +, residues suggested to interact with RNA polymerase (43); and =, amino acid residues probably involved in dimerization (30). The predicted helix-turn-helix motif is boxed and marked by a solid line above the sequence. Two amino acid residues in this DNA-binding region (Glu-209 and Ser-212) which are essential for binding to the FNR box (43) are shown in boldface.

and one internal cysteine residue (Cys-20, Cys-23, Cys-29, and Cys-122) are essential for the function of the protein in response to anaerobiosis (43). These cysteine residues are assumed to bind a $[4Fe—4S]^{2+}$ cluster which in vitro is converted to a $[2Fe—2S]^{2+}$ cluster by oxygen, resulting in the inactivation of FNR (22, 48). In ANR_{Pfl} as well as in ANR_{Pae} (66), these cysteines are conserved, with exactly the same spacing as in FNR (Fig. 5). A domain involved in the interaction of FNR with RNA polymerase (Ile-81, Thr-82, Gly-85, Asp-86, Glu-87, and Gln-88) (43) is similar but is less strictly conserved in ANR_{Pfl} and in ANR_{Pae} (Fig. 5). FNR binds to its target DNA sequence in a dimeric state, which is favored by low oxygen availability (48). Four amino acids (Asp-22, Leu-28, Glu-150, and Asp-154) which appear to be involved in dimerization and stabilization of the Fe—S cluster (30) are strictly conserved in both ANR_{Pfl} and ANR_{Pae} (Fig. 5). Finally, the C-terminal helix-turn-helix DNA-binding motifs are identical in ANR_{Pfl} and ANR_{Pae} (Fig. 5).

Construction and properties of a *P. fluorescens anr* mutant. The *anr* gene of strain CHA0 was disrupted by insertion of an Ω-Km element and introduced into the CHA0 chromosome by use of the suicide vector pME3087 (see Materials and Methods), resulting in the *anr* mutant CHA21 (Fig. 4). The phenotypic properties of the mutant were compared to those of the wild-type strain CHA0 and the complemented mutant CHA21/pME3817 (Fig. 4). The *anr* mutant had a strongly reduced ability to synthesize HCN, as predicted, and contained noninduced levels of arginine deiminase, the first enzyme of an arginine catabolic ATP-generating pathway (Table 3).

TABLE 3. Effect of an *anr* mutation on HCN production and arginine deiminase activity in *P. fluorescens*

Strain	HCN production[a] (μM)	Arginine deiminase sp act[b] (U/mg of protein)[c]
CHA0	89 ± 5	22 ± 3
CHA21	6 ± 3	3 ± 1
CHA21/pME3817	170 ± 44	29 ± 2

[a] Mean ± standard deviation for four experiments in MMC.
[b] Mean ± standard deviation for three experiments in YEA medium.
[c] One unit of activity was defined as 1 μmol of citrulline produced per h.

In *P. aeruginosa*, the *anr* gene is essential for anaerobic growth on nitrate by denitrification and on arginine by the arginine deiminase pathway (17, 64, 66). Therefore, *P. fluorescens* CHA0 was tested for growth in an anaerobic jar (GasPak). However, this strain did not grow anaerobically on arginine (YEA plates), on nitrate (NA amended with 20 mM KNO_3), or on nitrite (NA amended with 5 mM KNO_2) at 30°C within 4 days. The control strain *P. aeruginosa* PAO1 grew on these media. Furthermore, *P. fluorescens* CHA0 did not produce gas in unshaken nitrate broth in 2 days. Thus, in these tests, strain CHA0 behaved as a strict aerobe, and no difference was apparent between the wild-type strain and the *anr* mutant.

Provided that the arginine deiminase pathway functions properly in *P. fluorescens* CHA0, the wild-type strain gains ATP from arginine but the *anr* mutant does not during oxygen limitation. In a competition experiment, strain CHA0 gradually displaced the *anr* mutant CHA21 when both strains were incubated in rich arginine (YEA) medium with limiting oxygen for 2 days (Table 4). Complementing plasmid pME3817 (anr^+) largely protected the mutant (Table 4). Thus, the wild-type strain clearly benefits from *anr* function under such conditions.

Regulation of *hcn* expression by oxygen limitation. We confirmed that the ANR-dependent expression of the *hcn* genes was regulated by oxygen limitation in *P. fluorescens*. An *hcnA′-′lacZ* translational fusion in plasmid pME3219 (Table 1) was introduced into strains CHA0 and CHA21. On X-Gal plates, strain CHA0/pME3219 formed blue colonies, whereas strain CHA21/pME3219 remained white (corresponding to ≤5 Miller units of β-galactosidase), indicating that ANR is needed to drive the expression of the *hcn* genes. When strain CHA0/pME3219 was grown in MMC with good aeration, the *hcnA′-′lacZ* fusion gave low β-galactosidase activity (570 ± 20 Miller

TABLE 4. Competition between the wild-type strain CHA0 and the *anr* mutant CHA21 (Km^r), with and without complementing plasmid pME3817, in YEA medium[a]

Incubation (h)	% Km^r colonies in mixtures of:	
	CHA0 and CHA21[b]	CHA0 and CHA21/pME3817[c]
0	56	59
1	57	57
15	47	45
24	32	45
36	4	46
48	3	49

[a] Pairs of competing strains were set up by mixing overnight cultures 1:1 (by volume) and diluting the mixtures 50-fold with fresh YEA medium (60 ml) in 120-ml flasks. Incubation of the tightly closed flasks at 30°C resulted in oxygen-limited stationary-phase cultures (approximately 10^9 cells/ml) after 10 to 15 h.
[b] Mean values of triplicate experiments.
[c] Mean values of duplicate experiments.

TABLE 5. Effect of an *anr* mutation in *P. fluorescens* CHA0 on the protection of tobacco against black root rot caused by *T. basicola* in a water-saturated soil under gnotobiotic conditions

Microorganism added[a]		Plant fresh wt[b] (mg)	Root fresh wt[b] (mg)	% Root surface infected[b,c]	*P. fluorescens*[b] (log CFU/g of root)
P. fluorescens	*T. basicola*				
None	−	677 a	224 a	0 a	ND
CHA0 (wild type)	−	681 a	250 a	0 a	8.58 a
CHA21 (*anr*)	−	616 a	240 a	0 a	8.41 a
CHA21/pME3817 (*anr*$^+$)	−	621 a	239 a	0 a	8.59 a
None	+	151 d	32 d	83 d	ND
CHA0 (wild type)	+	450 b	152 b	31 b	8.98 a
CHA21 (*anr*)	+	327 c	97 c	58 c	9.13 a
CHA21/pME3817 (*anr*$^+$)	+	479 b	175 b	39 b	8.80 a

[a] *P. fluorescens* strains and *T. basicola* were added at 10^7 CFU and 5×10^3 endoconidia per g of soil 3 and 2 days, respectively, before planting of a 5-week-old tobacco plant grown under sterile conditions. Water-saturated conditions (water content, 35%; water potential, 0 MPa) were induced 3 days after planting by adding sterile water to soil. Plants were harvested after 3 weeks.

[b] Means within the same column followed by the same letter do not differ significantly at $P = 0.05$, according to Student's t test. Data represent three individual repetitions of the same experimental setup, with eight replicates (flasks containing one tobacco plant) per treatment. ND, not detected.

[c] Percentage of root surface darkened by the presence of chlamydospores of *T. basicola* (23).

units). After growth under oxygen limitation, the same strain expressed an elevated level of β-galactosidase activity (18,000 ± 2,000 Miller units), demonstrating control by microaerophilic conditions.

Effect of an *anr* mutation on the ability of *P. fluorescens* to suppress black root rot of tobacco. Suppression of root diseases by strain CHA0 depends to a large extent on antibiotic and HCN production (52). In vitro, the HCN-deficient *anr* mutant CHA21 produced the antibiotics 2,4-diacetylphloroglucinol (5.9 μg/ml) and pyoluteorin (15.5 μg/ml) in quantities that were comparable to those excreted by the wild-type strain CHA0 (7.2 and 9.9 μg/ml, respectively). Previously, we showed that, in a gnotobiotic system, the *hcnB*::Ω-Hg mutant CHA5, constructed by gene replacement, protects tobacco roots less effectively from the black root rot fungus *T. basicola* than does the wild-type strain CHA0. Moreover, *hcn* function and suppressive ability can be restored by complementation with pME3013 (53). We verified that the *hcn* deletion strain CHA77 gave reduced (84%) disease suppression in the tobacco-*T. basicola* system, in comparison with the wild-type strain CHA0 (disease suppression defined as 100%) and the complemented mutant CHA77/pME3013 (disease suppression, 110%), in terms of fresh plant weight (data not shown). The artificial soil used in these studies contained 23% water (soil water potential, −0.0028 MPa). The suppressive capacity of the *anr* mutant CHA21 in artificial soil containing 20% water (−0.004 MPa) was affected similarly (data not shown). In a wetter soil, containing 35% water (soil water potential, 0 MPa), under otherwise identical conditions, the *anr* mutant CHA21 afforded significantly reduced protection of tobacco, in terms of both plant weight and reduction of disease severity, in comparison with the wild-type strain CHA0 and the complemented mutant CHA21/pME3817 (Table 5). These data suggest that *anr* function can contribute to biocontrol, especially in soil with restricted oxygen availability.

DISCUSSION

Structure and function of HCN synthase. The nucleotide sequence of HCN synthase, established here for the first time, supports one of several models proposed for bacterial HCN synthesis (26): the dehydrogenase model. According to this mechanism, glycine is first oxidized to iminoacetic acid [H-C(NH)-COOH]. Then, the C—C bond is split, with a concomitant second dehydrogenase reaction, which produces HCN and CO_2 (58). The three HCN synthase subunits have similarities with known dehydrogenases: HcnA with a clostridial formate dehydrogenase and HcnB and HcnC with amino acid dehydrogenases (oxidases). All in all, the sequence comparisons strongly suggest that HCN synthase basically is an amino acid oxidase. By analogy with biochemically characterized amino acid oxidases (10, 35), it is predicted that HCN synthase is a flavoenzyme.

However, HCN synthase differs from D- and L-amino acid oxidases in an important aspect. The latter enzymes produce α-imino acids from their amino acid substrates; α-imino acids are rapidly hydrolyzed, giving the corresponding α-keto acids (20). In the HCN synthase reaction, however, the postulated intermediate iminoacetic acid does not appear to be converted hydrolytically to glyoxylic acid (26). Instead, enzyme-bound iminoacetic acid is assumed to be cleaved at the C—C bond.

This cleavage may have some similarity with the radical mechanism involved in the pyruvate-formate lyase (Pfl) reaction in *E. coli* (24). Cleavage of pyruvate is initiated by one-electron transfer from activated Pfl, which contains a free glycyl radical in the polypeptide chain. The glycine radical lies in a turn stretch (Val-Ser-Gly-Tyr) between two β strands in the C-terminal part of Pfl (55). The occurrence of a similar motif (Val-Glu-Gly-Tyr) forming a turn in the C-terminal region of HcnC (Fig. 2) may be fortuitous. However, a related sequence (Val-Cys-Gly-Tyr) is also found in anaerobic ribonucleotide reductase; at this site, a glycine radical is formed during activation of this enzyme (45). Molecular oxygen readily reacts with the glycine radical of Pfl, cleaving the polypeptide chain at this site (55). Perhaps the exquisite sensitivity of HCN synthase to oxygen (7, 60) could be explained by an analogous reaction.

Cleavage of iminoacetic acid by HCN synthase could produce HCN and formic acid, whose oxidation to CO_2 might be catalyzed by the HcnA subunit. The four electrons removed from glycine by HCN synthase are probably transferred to a cyanide-insensitive terminal oxidase; the CioAB enzyme recently characterized for *P. aeruginosa* (11) is a good candidate. Two transmembrane segments are predicted for both HcnB and HcnC (Fig. 2), suggesting that HCN synthase is a membrane-bound enzyme. These features correlate with previous enzyme data. Using a partially purified HCN synthase preparation, Wissing (58, 59) obtained evidence for FAD as a cofactor and for an association of the enzyme with the cytoplasmic membrane.

ANR function in *P. fluorescens*. For a wide variety of bacteria, more than 20 FNR homologs have been identified, most of

which control gene expression in response to oxygen limitation (43, 50, 62). According to the phylogenetic tree of the FNR family proposed by Van Spanning et al. (50), ANR_{Pfl} belongs to group A, where it resembles most ANR_{Pae} (88% identity) and FnrA of *P. stutzeri* (84% identity) and is more distantly related to FNR in *Enterobacteriaceae*. The FNR-like regulator CydR of *Azotobacter vinelandii* (62) is also a close relative of ANR_{Pfl} (80% identity).

In *P. aeruginosa* PAO, the *anr* gene mediates anaerobic growth on nitrate and on arginine (17, 66). We recently studied the function of ANR as a transcriptional regulator in this bacterium (57). Promoters containing the FNR consensus box are induced about 20-fold by oxygen limitation, compared to the basal levels measured in well-aerated cells (57). In *P. fluorescens* CHA0, which does not grow anaerobically by denitrification or by arginine fermentation, the *anr* gene is nevertheless conserved and physiologically active. In particular, the FNR consensus promoter shows a similar ANR-dependent response to oxygen (21a). The wild-type strain CHA0 produces about 15 times more HCN than does the *anr* mutant strain CHA21 under conditions of oxygen limitation (Table 3). Oxygen limitation was shown to regulate the expression of an *hcnA′-′lacZ* translational fusion in strain CHA0. The level of β-galactosidase was 30 times higher in oxygen-limited cultures than in cells grown with good aeration. Moreover, the fusion was not expressed in the *anr* mutant. From the *hcnA* promoter sequence (Fig. 2) it can be predicted that ANR binds to the −40 region and thereby activates transcription.

Mutational inactivation of the *anr* gene reduced the biocontrol efficacy of *P. fluorescens* CHA0 in the tobacco-*T. basicola* system (Table 5). This effect is in agreement with previous work on the role of bacterial HCN in this plant-pathogen system (53). The *anr* function of strain CHA0 seemed to have a stronger positive effect on biocontrol in water-saturated soil than in more aerated soil, suggesting that ANR-dependent HCN production occurs predominantly in poorly aerated, water-soaked soils. The site(s) where GacA regulates HCN synthesis (29) has not yet been determined.

Although *P. fluorescens* CHA0 does not grow in the anaerobic atmosphere generated by the GasPak jar, it can adapt to microaerobic conditions by using ANR as a positive regulator. Natural habitats of *P. fluorescens* often contain little oxygen. In biofilms formed by *P. fluorescens*, the amount of oxygen available to cells in the lower layers is only a small percentage of the amount present at the surface (36). In the rhizosphere, oxygen consumption by roots and root-colonizing microorganisms can create a marked oxygen gradient. In a barley root model system, the innermost zone around the root, the rhizoplane, contains only 1 to 20% of the oxygen concentration that is present in the outer zones, lying 3 mm from the root surface (21). The oxygen concentration needed to activate ANR of *P. fluorescens* has not been determined. However, the fact that the *anr* gene can effectively restore an *fnr* mutant of *E. coli* suggests that ANR and FNR are functionally similar. Half-maximal induction of FNR-dependent promoters in *E. coli* occurs at about 5 μM O_2, i.e., at about 2.5% air saturation (48). Thus, oxygen levels in the rhizoplane may be sufficiently low to allow the activation of ANR in *P. fluorescens*. ANR conferred a selective advantage on *P. fluorescens* cells in a nutrient-rich environment (Table 4). Another obligate aerobe, *A. vinelandii*, uses the FNR-like activator CydR for microaerobic growth (62). Thus, positive control of gene expression under microaerobic conditions may be quite common in aerobic bacteria having FNR-like regulators.

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The first and second authors contributed equally to this study.

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