JOURNAL OF BACTERIOLOGY, Dec. 2000, p. 6940–6949 Vol. 182, No. 24
0021-9193/00/$04.00+0

Transcriptional Control of the Hydrogen Cyanide Biosynthetic Genes *hcnABC* by the Anaerobic Regulator ANR and the Quorum-Sensing Regulators LasR and RhlR in *Pseudomonas aeruginosa*

GABRIELLA PESSI AND DIETER HAAS*

Laboratoire de Biologie Microbienne, Université de Lausanne, CH-1015 Lausanne, Switzerland

Received 8 June 2000/Accepted 30 August 2000

Virulence factors of *Pseudomonas aeruginosa* include hydrogen cyanide (HCN). This secondary metabolite is maximally produced at low oxygen tension and high cell densities during the transition from exponential to stationary growth phase. The *hcnABC* genes encoding HCN synthase were identified on a genomic fragment complementing an HCN-deficient mutant of *P. aeruginosa* PAO1. The *hcnA* promoter was found to be controlled by the FNR-like anaerobic regulator ANR and by the quorum-sensing regulators LasR and RhlR. Primer extension analysis revealed two transcription starts, T1 and T2, separated by 29 bp. Their function was confirmed by transcriptional *lacZ* fusions. The promoter sequence displayed an FNR/ANR box at −42.5 bp upstream of T2 and a *lux* box centered around −42.5 bp upstream of T1. Expression of the *hcn* genes was completely abolished when this *lux* box was deleted or inactivated by two point mutations in conserved nucleotides. The *lux* box was recognized by both LasR [activated by *N*-(oxododecanoyl)-homoserine lactone] and RhlR (activated by *N*-butanoyl-homoserine lactone), as shown by expression experiments performed in quorum-sensing-defective *P. aeruginosa* mutants and in the *N*-acyl-homoserine lactone-negative heterologous host *P. fluorescens* CHA0. A second, less conserved *lux* box lying 160 bp upstream of T1 seems to account for enhanced quorum-sensing-dependent expression. Without LasR and RhlR, ANR could not activate the *hcn* promoter. Together, these data indicate that expression of the *hcn* promoter from T1 can occur under quorum-sensing control alone. Enhanced expression from T2 appears to rely on a synergistic action between LasR, RhlR, and ANR.

Pseudomonas aeruginosa is a gram-negative bacterium that can cause serious infections in patients suffering from cystic fibrosis, cancer, infection with human immunodeficiency virus, or severe burn wounds (16, 29). The pathogenesis of *P. aeruginosa* infections is due to the production of both cell-associated and extracellular virulence factors. One of these extracellular compounds, hydrogen cyanide (HCN), has been found at relatively high concentrations in patients with freshly infected burns (27). Evidence for HCN being a virulence factor comes from an experimental infection model in which an *hcn* insertion mutant of *P. aeruginosa* had a strongly reduced ability to kill the nematode *Caenorhabditis elegans* (L. Gallagher and C. Manoil, Abstr. *Pseudomonas* '99: Biotechnol. Pathog., abstr. 75, 1999). Cyanide is a potent inhibitor of cytochrome *c* oxidase, the terminal component of the aerobic respiratory chain in many organisms, and of several other important metalloenzymes (52).

In *Pseudomonas* spp., HCN biosynthesis is catalyzed by the membrane-bound enzyme HCN synthase, which forms HCN and CO_2 from glycine (8). The enzyme is sensitive to molecular oxygen and has been purified only partially from a *Pseudomonas* species (60); hence, little is known about the biochemistry of the enzymatic reaction. In *P. fluorescens*, the structural *hcnABC* genes encoding HCN synthase are clustered and probably form an operon (34). From an analysis of nucleotide sequence data, it can be concluded that HCN synthase essentially functions as a glycine dehydrogenase/oxidase, transferring four electrons to a cyanide-resistant branch of the aerobic respiratory chain (5).

In *P. aeruginosa* and *P. fluorescens*, cyanogenesis proceeds at low oxygen concentrations (9, 11) and depends on ANR (anaerobic regulator of arginine deiminase and nitrate reductase), a transcriptional regulator, which is converted to its active form under low oxygen supply. ANR belongs to the FNR (fumarate and nitrate reductase regulator) family of transcriptional regulators (53); *anr* mutants of both species produce little HCN (34, 62). ANR can activate target promoters by binding to conserved sequences known as ANR boxes, with a recognition specificity which is similar but not identical to that of FNR (58).

Cell density is a second parameter that influences cyanogenesis in *Pseudomonas* spp. It was discovered 20 years ago that optimal expression of HCN synthase occurs during the transition from the exponential to the stationary phase (12). This expression pattern is characteristic of regulatory mechanisms which more recently have been termed quorum sensing (21). In *P. aeruginosa*, the production of virulence factors and secondary metabolites is under quorum-sensing control involving *N*-acyl-homoserine lactone signals (20). These signals are produced by an autoinduction mechanism with increasing cell density, and they are assumed to diffuse freely through the cell envelope. When reaching a threshold concentration, they activate LuxR-type transcriptional regulators which control the expression of target genes by recognizing conserved sequences termed *lux* boxes (17, 20). *P. aeruginosa* contains two interdependent quorum-sensing systems (43, 44, 57). In the *lasRI* system, the LasI protein directs the synthesis of *N*-(3-oxodo-

* Corresponding author. Mailing address: Laboratoire de Biologie Microbienne, Université de Lausanne, CH-1015 Lausanne, Switzerland. Phone: 41 21 692 56 31. Fax: 41 21 692 56 35. E-mail: Dieter.Haas@lbm.unil.ch.

decanoyl)-homoserine lactone (OdDHL), which triggers the transcriptional activator, LasR, to induce the expression of virulence factors such as elastases (LasB and LasA), exotoxin A, and alkaline protease (47). In addition, the *las* system autoregulates the *lasI* gene, leading to the production of more OdDHL (51), and upregulates the second quorum-sensing system, consisting of the transcriptional activator RhlR and the RhlI protein, which directs the synthesis of *N*-butanoyl-homoserine lactone (BHL). The *rhlRI* system regulates the production of multiple exoproducts including rhamnolipids, elastase (LasB), pyocyanin, and cyanide (7, 33, 57).

From previous studies, it appears that both autoinducers OdDHL and BHL contribute to the regulation of the *hcn* genes in *P. aeruginosa* (33, 45, 56, 57). However, it is not clear whether the OdDHL-LasR team can exert its effect on *hcn* gene expression directly or indirectly, i.e., by activating the *rhlRI* system. Furthermore, the question arises of how anaerobic control by ANR and quorum-sensing control by LasR and RhlR may cooperate during initiation of transcription of the *hcn* genes. In the present study, we have undertaken a systematic analysis of the *hcn* promoter region of *P. aeruginosa*. We show that the simultaneous action of three activators ANR, LasR, and RhlR ensures optimal expression of the *hcnABC* cluster.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* and *Escherichia coli* strains were routinely grown on nutrient agar plates and in nutrient yeast broth (NYB) with aeration at 37°C, whereas *P. fluorescens* strains were cultivated on these media at 30°C. For determination of HCN production, *P. aeruginosa* strains were grown with shaking at 180 rpm under mild oxygen limitation in 100-ml Erlenmeyer flasks containing 40 ml of a synthetic glycine minimal medium (MMC) described by Castric (10). Severe oxygen limitation was achieved by growing the cells in tightly closed 125-ml bottles containing 60 ml of MMC, with gentle shaking; at the end of growth, the oxygen present initially was consumed by the cells. Antibiotics were used at the concentrations (in micrograms per milliliter) indicated in parentheses: for *P. aeruginosa* strains, carbenicillin (250), chloramphenicol (250), gentamicin (10), tetracycline (125), and mercuric chloride (10); for *P. fluorescens*, gentamicin (10) and tetracycline (125); and for *E. coli*, ampicillin (100), chloramphenicol (25), gentamicin (10), and tetracycline (25). 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) was incorporated into solid media at 0.02% to monitor β-galactosidase expression (49).

DNA techniques and nucleotide sequencing. Small-scale preparations of plasmid DNA were carried out by the cetylmethylammonium bromide method (15), and large-scale preparations were performed using Qiagen-Tips (Qiagen). Chromosomal DNA was extracted from *P. aeruginosa* and purified as described elsewhere (24). Restriction enzyme digestions, ligations, and agarose gel electrophoresis were performed using standard procedures (49). Restriction fragments were purified from agarose gels using GeneClean II (Bio 101). Transformation of *E. coli*, *P. aeruginosa*, and *P. fluorescens* was carried out by electroporation (18). Southern blotting of *P. aeruginosa* DNA with Hybond N membranes (Amersham), random-primed DNA labeling of a 3.1-kb *Eco*RI-*Xho*I *hcnABC* fragment from pME3071 (Table 1) with digoxigenin-11-dUTP (Boehringer Mannheim), hybridization with this probe, and detection were all performed according to the protocols of the supplier. Nucleotide sequences of the *hcnA* gene with its upstream region and of the proximal 0.4 kb of the *hcnB* gene as well as of all PCR-derived constructs were determined by the dideoxy chain termination method using [^{35}S]dATP (Amersham), 7-deaza-dGTP, and Sequenase version 2.0 (United States Biochemical Corp.). Sequence analysis was performed using the Genetics Computer Group programs FASTA (for homology searches in the Genbank/EMBL and SwissProt databases), GAP, and BESTFIT (for comparisons of pairs of sequences).

Plasmid construction and mutagenesis. (i) Construction of a translational *hcnA′-′lacZ* fusion. The 0.78-kb *Eco*RV fragment from plasmid pME3336 containing the *hcn* promoter region and the first nine codons of the *hcnA* gene was fused in frame to the *′lacZ* fragment from pNM482, in pME6010, resulting in plasmid pME3826. The same *hcnA′-′lacZ* fusion was also cloned on a 3.88-kb fragment into vector pKT240, giving pME3823.

(ii) Construction of the deletion derivatives pME3832, pME3835, and pME3837. The deletion derivatives were obtained by PCR. Plasmid pME3826 was used as the template to amplify the respective *hcnA′* fragments by the use of primers homologous to positions −111 (5′-GATCGAATTCACCTACCAGAA TTGGCAGG-3′; pME3832), −88 (5′-GCTCGGATCCGATACCCACCTGTC ATGG-3′; pME3835), and −143 (5′-GCTCGGATCCGTTCGACTTTTCCGC GCG-3′, pME3837) and of a primer annealing within the *lacZ* sequence (5′-T GCTGCAAGGCGATTAAGTTGG-3′). The positions are given relative to the translational start (see Fig. 2B). These primers contain a restriction site (underlined) at the 5′ end: an *Eco*RI site for pME3832, and a *Bam*HI site for pME3835 and pME3837. For the amplification reactions, thermostable DNA polymerase (Eurobio) was used. Thermal cycling (15 cycles) consisted of denaturation at 95°C for 1 min, primer annealing at 58°C for 1 min, and elongation at 72°C for 1 min. The PCR fragments obtained were digested with *Eco*RV and *Eco*RI or *Bam*HI, cloned into pBluescript II SK, and sequenced. The deleted promoter regions were fused to the *′lacZ* gene (40) in pME6010, resulting in pME3832, pME3835, and pME3837 (see Fig. 6).

(iii) Site-directed mutagenesis of the *hcnA* promoter region. The two-base substitution in plasmid pME3844, which is otherwise identical to pME3837, was generated by the overlap extension method (39). Primer 5′-CCCAC**TT**ACCAG AATTGGCA**A**GGGAAG-3′ was used to generate the mutations (boldface) in the *lux* box (see Fig. 5). The mutation was verified by sequencing. An *Sph*I restriction site was introduced between the ANR box and the *lux* box by PCR, using as template pME3336, which contains the *hcn* promoter on a 0.78-kb *Eco*RV insert. The whole 3.78-kb plasmid was amplified using primers I1 (5′-GTCCGCATGCACCCACCTGTCATGGAT-3′) and I2 (5′-GTCCGCATGCA TCTTCCCCTGCCAATT-3′), each containing an *Sph*I restriction site (underlined). The PCR product was digested with *Sph*I and ligated. The 6-bp insertion was verified by sequencing, and the 0.78-kb *Eco*RV insert was fused in frame with the *′lacZ* reporter gene from pNM482 in vector pME6010, creating plasmid pME3852.

(iv) Construction of pME3850.1 and pME3850.2 containing a transcriptional *lacZ* fusion. Plasmids pME3850.1 and pME3850.2 were obtained by PCR amplification of the *hcn* promoter regions using an *Eco*RI-tagged primer (the *Eco*RI site is underlined), homologous to position −188 (5′-GCTCGAATTCACGGG TGAGCCGGC-3′), and two primers which anneal at the +1 transcriptional start sites T1 (5′-GCTCCTGCAGGCGGACATTGATCC-3′ for pME3850.1) and T2 (5′-GCTCCTGCAGAGCCGGCGACACTAG-3′ for pME3850.2) and which each create an artificial *Pst*I site (underlined). The 136- and 165-bp *Eco*RI/*Pst*I-digested PCR fragments were fused to the *lacZ* gene of pME6522, resulting in pME3850.1 and pME3850.2, respectively.

Construction of *P. aeruginosa* mutant strains. The chromosomal *hcnA′-′lacZ* reporter strains PAO6289 (*hcnA′-′lacZ*), PAO6290 (*anr hcnA′-′lacZ*), PAO6293 (*rhlR hcnA′-′lacZ*), and PAO6326 (*lasR hcnA′-′lacZ*) were obtained as follows. The translational *hcnA′-′lacZ* fusion from plasmid pME3826 was cloned into the suicide plasmid pME3087ΔE. The resulting plasmid pME3825 was mobilized by the helper plasmid pRK2013 into the different derivatives of *P. aeruginosa* and chromosomally integrated with selection for tetracycline resistance (Tc^r). Excision of the vector by a second crossover was obtained by enrichment for tetracycline-sensitive (Tc^s) cells (61). The chromosomal insertions of the *hcnA′-′lacZ* translational fusion were checked by Southern blotting (data not shown) and by testing their β-galactosidase-positive phenotype. The *P. aeruginosa rhlR lasI* double-mutant strain PAO6351 was constructed by transduction. The transducing phage E79*tv*2 (41) was propagated on strain PAOJP1 and then used to transduce the *lasI*::Tc^r mutation into the *rhlR* mutant strain PDO111. The transductants were selected on nutrient agar plates containing tetracycline and mercuric chloride.

RNA isolation and transcriptional start site mapping. Extraction of total RNA from *P. aeruginosa* cells grown in MMC (for conditions, see the legend to Fig. 4) was performed by a single-step RNA isolation method (14) using the TRIzol reagent (GIBCO-BRL). After treatment with amplification-grade DNase (Pharmacia), 10-μg aliquots of the isolated RNA were analyzed by primer extension as described elsewhere (54). The oligonucleotide P_E 5′-GAGGTGGAT GGTCATGTCTGCCCGCGAGAG-3′ (positions +65 to +36), which anneals to the coding strand of *hcnA*, was 5′-end labeled with 20 μCi of [γ-^{32}P]dATP (Amersham) and 10 U of T4 polynucleotide kinase (Pharmacia) at 37°C for 30 min; 0.05 pmol of the [^{32}P]dATP-labeled primer, purified with a nucleotide removal kit (Qiaquick; Qiagen) was dissolved in 20 mM Tris-HCl (pH 8.3)–200 mM NaCl–0.1 mM EDTA together with 10 μg of total RNA in a final volume of 30 μl. The solution was boiled for 3 min and incubated at 60°C for 2 h, then at 37°C for 30 min, and at room temperature for 30 min. Avian myeloblastosis virus reverse transcriptase (30 U; Pharmacia) was used to extend the primer in a reaction mixture containing 94 mM Tris-HCl (pH 8.3), 10 mM $MgCl_2$, 10 mM dithiothreitol, 45 U of RNase inhibitor (Pharmacia), and 0.5 mM (each) deoxynucleoside triphosphate. The reaction was carried out in a total volume of 100 μl at 42°C for 1 h. After phenol-chloroform extraction, the extended product was precipitated by the addition of 0.1 volume of 3 M sodium acetate and 3 volumes of ethanol. The pellet was washed with 75% ethanol, dried, and redissolved in 10 μl of H_2O. The unlabeled primer was used to generate a nucleotide sequence ladder using a T7 sequencing kit (Pharmacia) with [^{35}S]dATP. Primer extension products were separated in an 8 M urea–8% polyacrylamide gel, in parallel with the sequencing reactions to map the transcription initiation sites.

Assay for regulation by LasR and RhlR in a heterologous background. To develop an RhlR/BHL assay in *P. fluorescens* CHA0, we cloned the 2-kb *Pst*I fragment containing the *rhlRI′* genes (33) behind the *lac* promoter of the vector pME6001, producing pME3840. Although the *rhlI* gene lacks nine codons at the 3′ end in this construct, BHL synthase activity was not affected. For a LasR/OdDHL assay, the 1.1-kb *Pvu*I fragment containing *lasR* (23) was similarly expressed from the *lac* promoter of vector pME6001, resulting in pME3827. The

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Genotype or description; phenotype	Reference or origin
Strains		
P. aeruginosa		
PAO1	Wild type	30
PAO2324	*met-9020 catA1 nar-9011 tyu-9009 puuD6 hcn*	36
PAO6261	Δ*anr*	61
PAO6289	*hcnA′-′lacZ*	This study (Fig. 2)
PAO6290	Δ*anr hcnA′-′lacZ*; PAO6261 derivative	This study
PAO6293	*rhlR*::Tn*501 hcnA′-′lacZ*; Hg^r; PDO111 derivative	This study
PAO6326	*lasR*::pUC19 (carrying *′lasR′* on a 0.4-kb *Pst*I fragment) *hcnA′-′lacZ*; Cb^r; PAOR derivative	This study
PAO6330	Δ*lasRI*	C. Reimmann, unpublished
PAO6351	*rhlR*::Tn*501* derivative of PAOJP1; Hg^r Tc^r	This study
PAOJP1	Δ*lasI*::Tc^r; Tc^r	43
PAOJP2	Δ*lasI*::Tc^r Δ*rhlI*::Tn*501*; Tc^r Hg^r	43
PAOR	*lasR*::pUC19 (carrying *′lasR′* on a 0.4-kb *Pst*I fragment); Cb^r	32
PDO111	*rhlR*::Tn*501*; Hg^r	7
P. fluorescens CHA0	Wild type	55
E. coli DH5α	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 deoR* Δ(*lacZYA-argF*)U169 λ-(ϕ 80d*lacZ*ΔM15)	49
Plasmids		
pBluescript II KS	Cloning vector, ColE1 replicon; Ap^r	Stratagene
pBBR1MCS	Broad-host-range cloning vector; Cm^r	31
pJF118EH	Cloning vector, ColE1 replicon, *lacI*^{q}, p*tac*; Ap^r	22
pKT240	Cloning vector, IncQ replicon, Mob; Ap^r Km^r	3
pME3071	pMMB67EH with a 5-kb *Xho*I-*Hin*dIII fragment of CHA0 containing *hcnABC′*	34
pME3087ΔE	Suicide vector, ColE1 replicon, Mob; *Eco*RI site deleted; Tc^r	59
pME3326	pBBR1MCS with a 6.6-kb *Bam*HI fragment of PAO1 containing the *hcnABC* region	This study
pME3333	pBBR1MCS with a 4.25-kb *Sal*I-*Xba*I fragment of PAO1 containing the *hcnABC* genes	This study (Fig. 2A)
pME3336	pBluescript with a 0.78-kb *Eco*RV fragment of PAO1 containing the *hcn* promoter region and the first 9 codons of *hcnA*	This study
pME3823	pKT240 with a 0.78-kb *Eco*RV *hcnA* upstream fragment and a translational *hcnA′*::*′lacZ* fusion at the *Eco*RV site in *hcnA*	This study
pME3825	pME3087Δ*Eco*RI with a 4.8-kb *Eco*RV-*Sma*I fragment containing a translational *hcnA′*::*′lacZ* fusion	This study (Fig. 2C)
pME3826	pME6010 with a 0.78-kb *hcnA* upstream fragment and a translational *hcnA′*::*′lacZ* fusion at the *Eco*RV site in *hcnA*	This study
pME3827	pME6001 carrying *lasR* on a 1.1-kb *Pvu*I-*Kpn*I fragment; Gm^r	This study
pME3832	pME6010 with the 111-bp *hcnA* upstream fragment and a translational *hcnA′*::*′lacZ* fusion at the *Eco*RV site in *hcnA*	This study (Fig. 6)
pME3835	pME6010 with the 88-bp *hcnA* upstream fragment and a translational *hcnA′*::*′lacZ* fusion at the *Eco*RV site in *hcnA*	This study (Fig. 6)
pME3837	pME6010 with the 143-bp *hcnA* upstream fragment and a translational *hcnA′*::*′lacZ* fusion at the *Eco*RV site in *hcnA*	This study (Fig. 6)
pME3840	pME6001 carrying *rhlRI′* on a 2-kb *Pst*I fragment; Gm^r	This study
pME3844	Derivative of pME3837 carrying 2 point mutations	This study (Fig. 6)
pME3850.1	pME6522 with 130 bp upstream of transcriptional start site T1	This study (Fig. 2D)
pME3850.2	pME6522 with 159 bp upstream of transcriptional start site T2	This study (Fig. 2D)
pME3852	Derivative of pME3826 with 6-bp insertion specifying an *Sph*I site	This study (Fig. 6)
pME6000	Cloning vector derived from pBRR1MCS; Tc^r	37
pME6001	Gm^r derivative of pME6000	6
pME6010	pACYC177-pVS1 shuttle vector; Tc^r	28
pME6522	pME6010 containing the promoterless *lacZ* gene on a 3.3-kb *Pst*I/*Dra*I fragment	6
pMJG1.7	pSW200 carrying the *lasR* gene on a 1.7-kb *Eco*RI-*Sac*I fragment of *P. aeruginosa* PAO1; Ap^r	23
pMMB67EH	Cloning vector, IncQ replicon, *lacI*^{q}, p*tac*; Ap^r	22
pMP21	pMMB190 carrying the *rhlRI′* genes on a 2-kb *Pst*I fragment; Ap^r	33
pNM482	ColE1 replicon; *′lacZ*; Ap^r	40
pRK2013	Helper plasmid, ColE1 replicon, Tra; Km^r	18

two plasmids obtained were electroporated separately into *P. fluorescens* strain CHA0, which additionally contained an *hcnA′-′lacZ* translational fusion of *P. aeruginosa* on plasmid pME3837 or pME3844. To examine the effect of the *las* or *rhl* autoinduction system on *hcnA* expression, we measured β-galactosidase activities.

HCN production. HCN was quantified in *P. aeruginosa* culture supernatants as described previously (26). Strains growing on plates were tested qualitatively for HCN production by an indicator paper method (9).

Assay of β-galactosidase activity. *Pseudomonas* cells were routinely grown in 100-ml flasks containing 40 ml of MMC with shaking at 180 rpm. β-Galactosidase specific activities were determined by the Miller method (49).

Autoinducer estimation by thin-layer chromatography. Cultures (200 ml) of strains PAO1, PDO111, PAO6261, PAO6330, PAOJP1, PAOJP2, PAO6351, CHA0, and CHA0 containing pME3840 were grown in NYB at 37°C (*P. aeruginosa*) or at 30°C (*P. fluorescens*) with shaking to an optical density at 600 nm (OD_{600}) of 1.7 to 2.0. Cells were removed by centrifugation (10,000 rpm for 10 min), and the cell-free supernatants were adjusted to pH 5.0 prior to extraction with an equal volume of dichloromethane in a separating funnel. The solvent phase was treated with anhydrous $MgSO_4$ to eliminate H_2O and evaporated to dryness using a rotary evaporator. The total extract was concentrated 200-fold by dissolving it in 1 ml of 50% (vol/vol) acetonitrile and was stored at −20°C. The presence of *N*-acyl-homoserine lactones in 1 to 5 μl of extract was tested by C_{18}

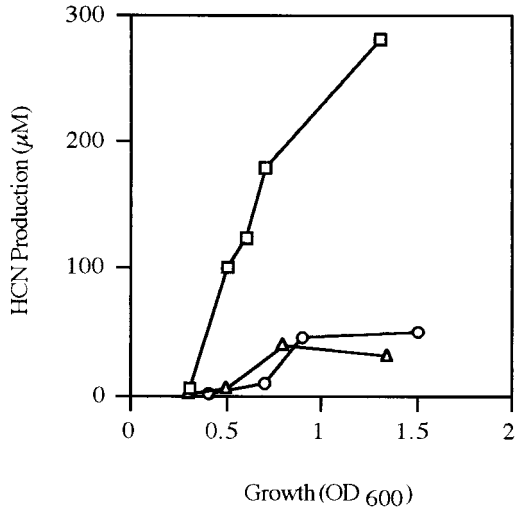

FIG. 1. Cell density-dependent HCN production in *P. aeruginosa*. The wild type PAO1 (□), the *anr* mutant PAO6261 (○), and the *rhlR* mutant PDO111 (△) were grown with oxygen limitation in 40 ml of MMC at 37°C. HCN was measured as described in Materials and Methods. Each point is the mean of three independent experiments.

reverse-phase thin-layer chromatography, developed with methanol-water (60:40, vol/vol), and revealed by the indicator organisms, *Chromobacterium violaceum* mutant CV026 (38) for BHL and *Agrobacterium tumefaciens* (*traG-lacZ*) (13) for OdDHL. By comparison with known amounts of BHL and OdDHL standards, we estimated that *P. aeruginosa* wild-type strain PAO1 and the *anr* mutant PAO6261 produced about 5 μM BHL and 2 μM OdDHL. The *rhlR* mutant strain PAO6293 showed about 2 μM each BHL and OdDHL. Strains PAO6330 and PAOJP1 were defective for the production of OdDHL and produced eight times less BHL than did the wild type. Strain PAOJP2 was completely defective for both autoinducers, while the *rhlR* strain PAO6351 produced only 2 μM BHL. The introduction of plasmid pME3840, which contains the *rhlR* gene and almost the whole *rhlI* gene, resulted in the production of 2 μM BHL in *P. fluorescens* strain CHA0; without this plasmid, neither BHL nor OdDHL was detectable.

Nucleotide sequence accession number. The sequence of the complete *hcnABC* cluster of *P. aeruginosa* is available under GenBank accession number AF208523 and at http://www.pseudomonas.com.

RESULTS

Relative importance of ANR, LasR, and RhlR for cyanogenesis in *P. aeruginosa*. To assess quantitatively the contribution of each of the transcriptional regulators, we measured HCN formation in mildly oxygen limited *P. aeruginosa* cultures growing in MMC. In the wild-type strain PAO1, HCN production strongly depended on cell density (Fig. 1). Both an *anr* and an *rhlR* mutant excreted about six times less HCN than did the wild type, at 10^9 cells per ml (corresponding to an OD_{600} of 1.0) (Fig. 1). A *lasR* mutant did not produce any detectable quantities of HCN (data not shown). These results demonstrate that LasR is absolutely required for cyanogenesis and suggest that LasR, RhlR, and ANR do not act independently of each other.

Identification and characterization of the *P. aeruginosa hcnABC* genes. We used the *hcnABC* genes of *P. fluorescens* CHA0 (34) as a probe for Southern hybridization analysis to detect the homologous genes with their promoter in *P. aeruginosa* PAO1. A chromosomal 6.6-kb *Bam*HI fragment of strain PAO1 gave a hybridization signal and was inserted into the broad-host-range vector pBBR1MCS, resulting in pME3326. Subcloning of a 4.25-kb *Sal*I-*Xba*I fragment (Fig. 2A) into the same vector produced pME3333, which complemented the HCN-negative mutant PAO2324 for HCN production, as determined by a qualitative test. The nucleotide sequence of this fragment revealed three open reading frames showing 77% identity with the *hcnABC* genes of *P. fluorescens* CHA0. The order of the *hcn* genes and their organization as a putative operon are conserved in both *Pseudomonas* species. The deduced amino acid sequences of the HcnA, HcnB, and HcnC polypeptides of *P. aeruginosa* show 69, 70, and 76% identity, respectively, with the corresponding gene products of *P. fluorescens*. In particular, the [2Fe-2S] motif in HcnA and the dehydrogenase motifs in HcnB and HcnC were conserved. By contrast, the promoter region upstream of *hcnA* in *P. aeruginosa* (Fig. 2B) diverges from the *hcnA* promoter of *P. fluorescens* (34), showing only 40% identity on a stretch of 210 nucleotides. This suggests that there may be differences in the regulatory mechanisms that control *hcn* expression in these two species.

Inactivation of the chromosomal *hcnABC* genes of *P. aeruginosa* by deletion resulted in an HCN-negative phenotype (data not shown), confirming the function of these genes. A translational *hcnA′-′lacZ* fusion (Fig. 2C) was introduced by gene replacement into the chromosome of the wild type and the *anr*, *lasR*, and *rhlR* mutants. β-Galactosidase activities measured in the resulting PAO derivatives (Fig. 3) closely paralleled HCN levels (Fig. 1), confirming the regulatory roles of the three transcription activators in cyanogenesis.

Mapping and characterization of the *P. aeruginosa hcnA* promoter region. The region upstream of *hcnA* was mapped by primer extension using primer P_E, which anneals to positions +65 to +36 relative to the translational start (Fig. 2B). Total RNA was extracted from *P. aeruginosa* wild-type strain PAO1 grown in MMC under mild oxygen limitation to different cell densities (Fig. 4, lanes 1 and 2). Two *hcn* transcripts were found (Fig. 4), suggesting two transcriptional start sites, T1 and T2 (Fig. 2B). At high cell density, the upstream T1 transcript, which mapped to nucleotide −59 (relative to the *hcnA* start codon), was more abundant than was the downstream T2 transcript starting at nucleotide −30, whereas at low cell density the opposite transcript abundance was observed (Fig. 4). This suggests that quorum-sensing control might be particularly important for transcription from start site T1. In agreement with this interpretation, RNA isolated from the *rhlR* mutant PDO111 gave only the downstream T2 transcript (Fig. 4, lane 5). No attempt was made to map transcripts in a *lasR* mutant because in this background *hcn* gene expression was extremely low (Fig. 3). When RNA was extracted from the wild-type strain PAO1 grown under anaerobic conditions, only the downstream T2 transcript was detectable (Fig. 4, lane 3). Furthermore, this transcript was absent from the *anr* mutant PAO6261 (Fig. 4, lane 4). These data suggest that transcription starting at T2 is basically under the control of the anaerobic regulator ANR.

A potential ANR/FNR box (CTGTC. . . .ATCAA) was found centered at −42.5 bp from the transcriptional start site T2 (Fig. 2B). As shown below, this box is functional although it deviates in the left half-site from the ANR/FNR consensus box (TTGAT. . . .ATCAA) (53, 58). The right ANR half-site overlaps with the −10 sequence of the upstream promoter. Centered at −42.5 bp from the start site T1 of this promoter, there is a palindromic sequence resembling the *Vibrio fischeri lux* box (17, 21). This potential *lux* box (designated α in Fig. 2B) is very similar to the quorum-sensing-controlled operators of the *P. aeruginosa rhlI*, *lasB*, *rhlA*, and *phzA* promoters (1, 42, 43, 48, 56). An alignment (Fig. 5) shows that 13 out of 16 conserved nucleotides in a *P. aeruginosa* consensus *lux* box are also present in box α, which therefore could be recognized by LasR and/or RhlR. The postulated *lux* and ANR boxes in the

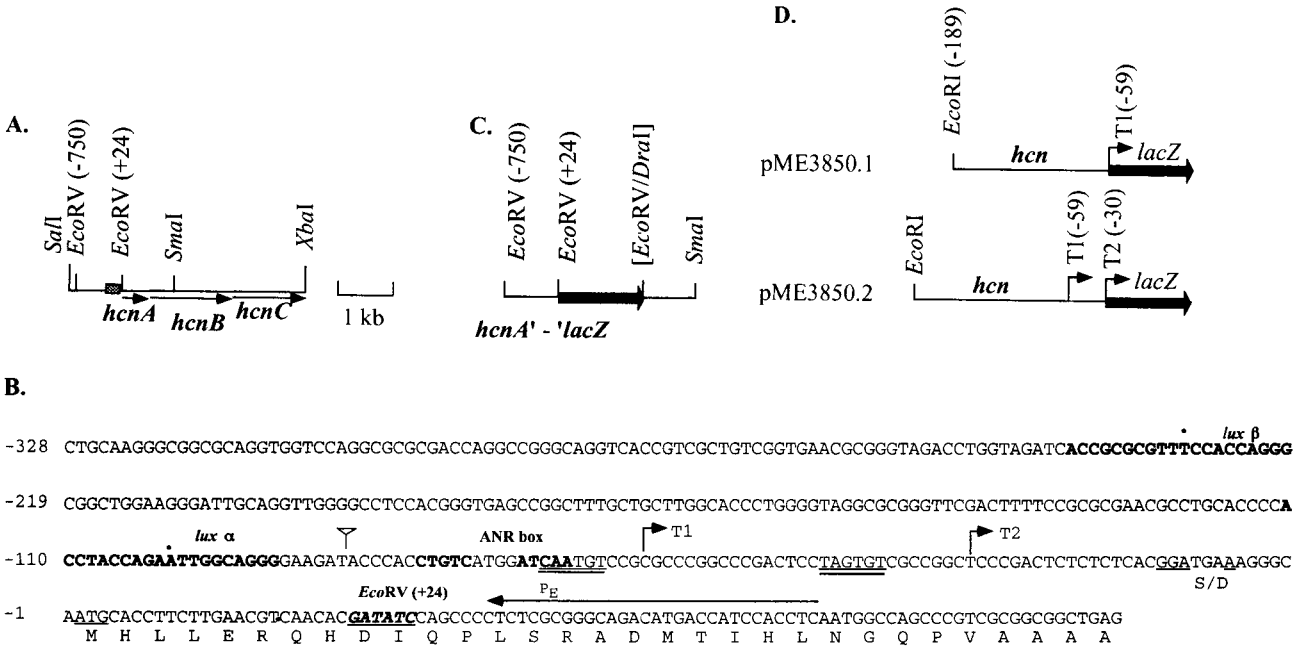

FIG. 2. The *hcnABC* cluster, its promoter region, and *lacZ* constructs. (A) The *hcnABC* biosynthetic genes (indicated by arrows) were cloned on a 4.25-kb *Sal*I/*Xba*I fragment into pBRR1MCS, giving pME3333. (B) The *hcnA* promoter region (indicated by ▩ in panel A) was mapped as shown in Fig. 4. Numbering is relative to the *hcnA* ATG start codon. The transcriptional start sites T1 and T2 are indicated by arrows. The putative translation initiation codon (deduced by comparison with the HcnA polypeptide of *P. fluorescens* [34]) and the potential ribosome binding site (S/D) are underlined. Double underlining indicates the −10 promoter regions. The ANR box and the palindromic *lux* boxes α and β (Fig. 5) are indicated in boldface. The primer (P_E) used in the primer extension experiment (Fig. 4) is indicated by an arrow above the sequence. The insertion of an *Sph*I restriction site in plasmid pME3852 (Fig. 6) is indicated by an inverted triangle. (C) A translational *hcnA'-'lacZ* fusion was integrated into the chromosome of the wild type and various mutants (Fig. 3) via homologous recombination. (D) PCR-amplified fragments, containing 130 bp upstream of T1 or 159 bp upstream of T2, were fused to the promoterless *lacZ* gene of pME6522, resulting in two transcriptional fusions, pME3850.1 and pME3850.2, respectively. The *Eco*RI site used was created by PCR.

hcn promoter both had a canonical distance of 42.5 bp from the respective transcription start sites, T1 and T2 (17, 53, 58). Interestingly, there is a second, less conserved *lux* box-like sequence (designated β in Fig. 2B) 108 bp upstream of *lux* box α (Fig. 5).

To confirm the function of the two promoters, P1 and P2, corresponding to the transcriptional starts T1 and T2, respectively, we constructed two transcriptional *lacZ* fusions. To this end, both promoters were fused precisely at their +1 transcription start sites to the *lacZ* reporter gene (Fig. 2D). The β-galactosidase activities of the resulting plasmids pME3850.1 and pME3850.2 were measured in *P. aeruginosa* wild type and in *anr*, *rhlR*, and *lasRI* mutants grown in MMC with mild oxygen limitation (Table 2). Plasmid pME3850.1, which contains only the quorum-sensing-dependent promoter P1, depended on RhlR and LasR for expression, whereas the presence or absence of ANR did not influence the activity significantly (Table 2). Plasmid pME3850.2, which contains both promoters, showed higher β-galactosidase activity and a clear dependence on ANR, LasR, and RhlR (Table 2). The impact of ANR appears to be greater on the translational *hcnA'-'lacZ* fusion (Fig. 3) than on the transcriptional *hcn* (P2)-*lacZ* fusion (Table 2), for unknown reasons. Under the conditions chosen (ca. 10^9 cells/ml), the promoter activity of P1 was slightly stronger than that of P2 (Table 2).

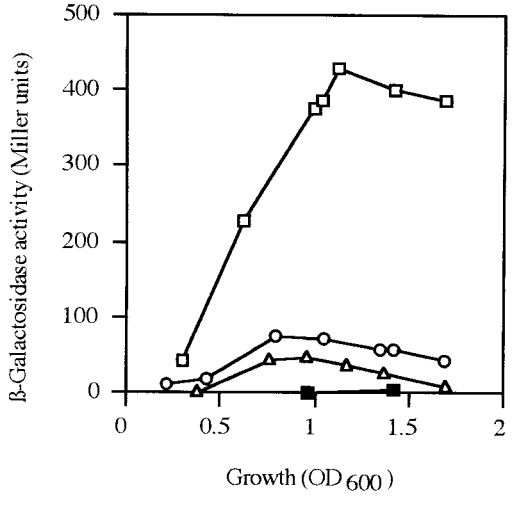

FIG. 3. Cell density-dependent expression of a translational *hcnA'-'lacZ* fusion in *P. aeruginosa*. Cultures of PAO6289 (□), the *anr* mutant PAO6290 (○), the *rhlR* mutant PAO6293 (△), and the *lasR* mutant PAO6326 (■) were grown with oxygen limitation in 40 ml of MMC at 37°C. Each β-galactosidase measurement is the mean of three independent experiments.

Mutational analysis of the *hcnA* promoter region. To explore further the role of the potential operator sequences associated with the *hcnA* promoter, we used a translational *hcnA'-'lacZ* reporter with the entire upstream region (in pME3826) (Fig. 6). Progressive deletions removed the upstream *lux* box β and neighboring sequences (in pME3837 and pME3832) and then the downstream *lux* box α (in pME3835). The ANR box was not deleted, as this would also have abolished the −10 sequence of the promoter P1. All constructs were assayed in the wild type and in the *anr*, *rhlR*, and *lasRI* mutants at a uniform cell density (10^9 cells/ml) under mild oxygen limitation. None of the constructs was active in the

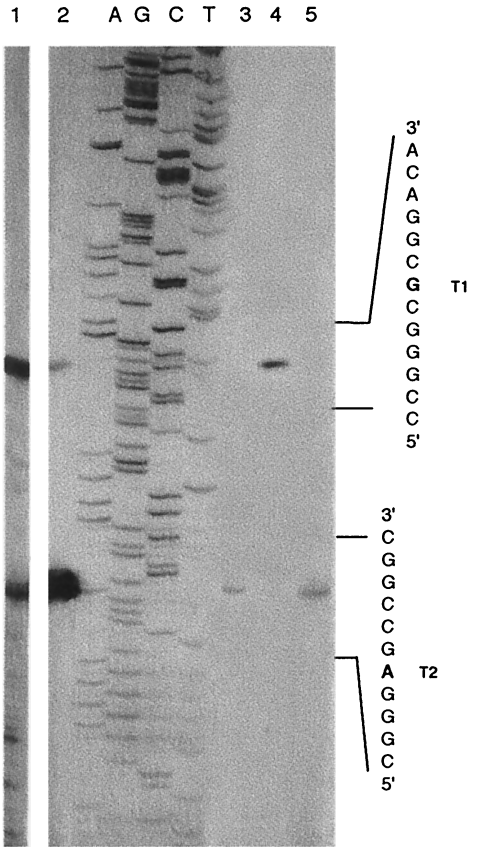

FIG. 4. Primer extension analysis of the 5′ end of the *hcnABC* transcripts. The 5′-end-labeled oligonucleotide P_E (Fig. 2B) was used as described in Materials and Methods. Lanes 1 and 2 show the extension products obtained with RNA from *P. aeruginosa* PAO1 grown semianaerobically to OD_{600} of 2.3 and 0.4, respectively, under the same conditions as for Fig. 1 and 3. Lane 3 contains the extension product of the *hcn* transcript from PAO1 grown to an OD_{600} of 0.9 under anaerobic conditions (in 60 ml of MMC; tightly closed 125-ml bottles). Lanes 4 and 5 show transcriptional start sites obtained with RNA from an *anr* mutant (PAO6261) and an *rhlR* mutant (PDO111), respectively, grown semianaerobically to an OD_{600} of 0.9. In lanes A, G, C, and T, the sequencing ladders obtained with the unlabeled oligonucleotide P_E were run in parallel. Because of the 5′ phosphate, the primer extension products move 0.5 nucleotide ahead of the corresponding band in the ladder.

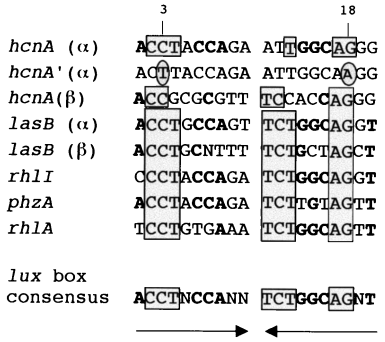

FIG. 5. Alignment of the *lux* boxes found in the *hcnABC* promoter region with other *lux* boxes of *P. aeruginosa*. The sequences shown are found in the promoters of four autoinducible genes, *rhlI* (42), *lasB* (48), *rhlA* (43), and *phzA* (GenBank accession number AF005404). A similar palindromic (→ ←) consensus sequence has been proposed previously by Whiteley et al. (56). Highly conserved nucleotides (six out of seven) are boxed, and less conserved nucleotides (five out of seven) are indicated in boldface. Two nucleotides that were mutated in the *hcnA′* α *lux* box of pME3844 are shown in ovals.

lasRI background (Fig. 6), confirming the essential role of the *las* system. Deletion of the *lux* box α or introduction of two point mutations into this box at conserved nucleotides (pME3844 [Fig. 5]) also prevented expression completely (Fig. 6). This indicated that *lux* box α is required for the functioning of both promoters and that ANR alone is insufficient to activate the promoter via its (imperfect) ANR box. The region lying upstream of *lux* box α enhanced expression about threefold; part of this effect could be due to the poorly conserved *lux* box β (Fig. 6). In the *anr* mutant PAO6261, constructs pME3826, pME3837, and pME3832 were expressed at levels about fivefold below the levels in the wild type, indicating that ANR acts as a downstream activator at the ANR box (Fig. 6). In the *rhlR* mutant PDO111, expression of the same constructs was 7 to 17 times lower than in the wild type PAO1 but above the background levels in the *lasRI* mutant PAO6330 (Fig. 6). These data indicate that LasR, together with either RhlR or ANR, can provide some expression, but that a synergy between all three regulators is necessary for maximal expression. Spacing between the *lux* box and the ANR box could be altered by a 6-bp insertion (in pME3852), without significant effects on expression (Fig. 6). In conclusion, the data in Table 2 and Fig. 4 and 6 show that quorum-sensing control operates on promoter P1 and, when boosted by ANR and low oxygen availability, also on promoter P2.

Both LasR and RhlR individually can activate the *hcnA* promoter. The *lasI rhlI* double mutant PAOJP2 expressed an *hcnA′-′lacZ* fusion poorly. Addition of both OdDHL and BHL restored β-galactosidase activity to near the wild-type level, whereas each autoinducer added singly had a smaller effect (Table 3), confirming the data of Whiteley et al. (56) obtained with an *hcnB-lacZ* fusion. Since LasR/OdDHL activates the expression of the *rhlR* and *rhlI* genes (32, 44) in the quorum-sensing cascade, the effect of OdDHL in strain PAOJP2 might have been indirect. Therefore, a *lasI rhlR* double mutant (PAO6351) was constructed. In this mutant, addition of OdDHL stimulated *hcnA′-′lacZ* expression about fivefold (Table 3). To confirm that LasR/OdDHL and RhlR/BHL can individually activate the *hcnA* promoters, we used *P. fluorescens* strain CHA0 as a heterologous host which does not synthesize *N*-acyl-homoserine lactones (6). (*E. coli* DH5α proved unsuitable for this experiment because the *hcnA* promoter was not expressed in this background.) In strain CHA0, the *P. aeruginosa hcnA′-′lacZ* fusion carried by pME3837 (Fig. 6) was expressed at a basal level (103 Miller units [Table 4]). Introduction of the *P. aeruginosa rhlRI* genes on pME3840 enhanced expression fourfold (Table 4). The production of BHL

TABLE 2. β-Galactosidase activities of two transcriptional *hcnA-lacZ* fusions constructed at T1 and T2 in *P. aeruginosa* wild type and regulatory mutants[a]

Strain (genotype)	β-Galactosidase activity (Miller units)	
	pME3850.1 (P1)	pME3850.2 (P1+P2)
PAO1 (wild type)	2,299 ± 259	3,971 ± 183
PAO6261 (*anr*)	1,887 ± 80	2,721 ± 111
PDO111 (*rhlR*)	186 ± 32	281 ± 4
PAO6330 (*lasRI*)	99 ± 1	98 ± 1

[a] Strains were grown in 40 ml of MMC in 100-ml Erlenmeyer flasks at 37°C with shaking to an OD_{600} of 1.0 and 1.2. β-Galactosidase activities were determined in triplicate; means ± standard deviations of three independent experiments are given. The *hcnA-lacZ* transcriptional fusion constructs are shown in Fig. 2D.

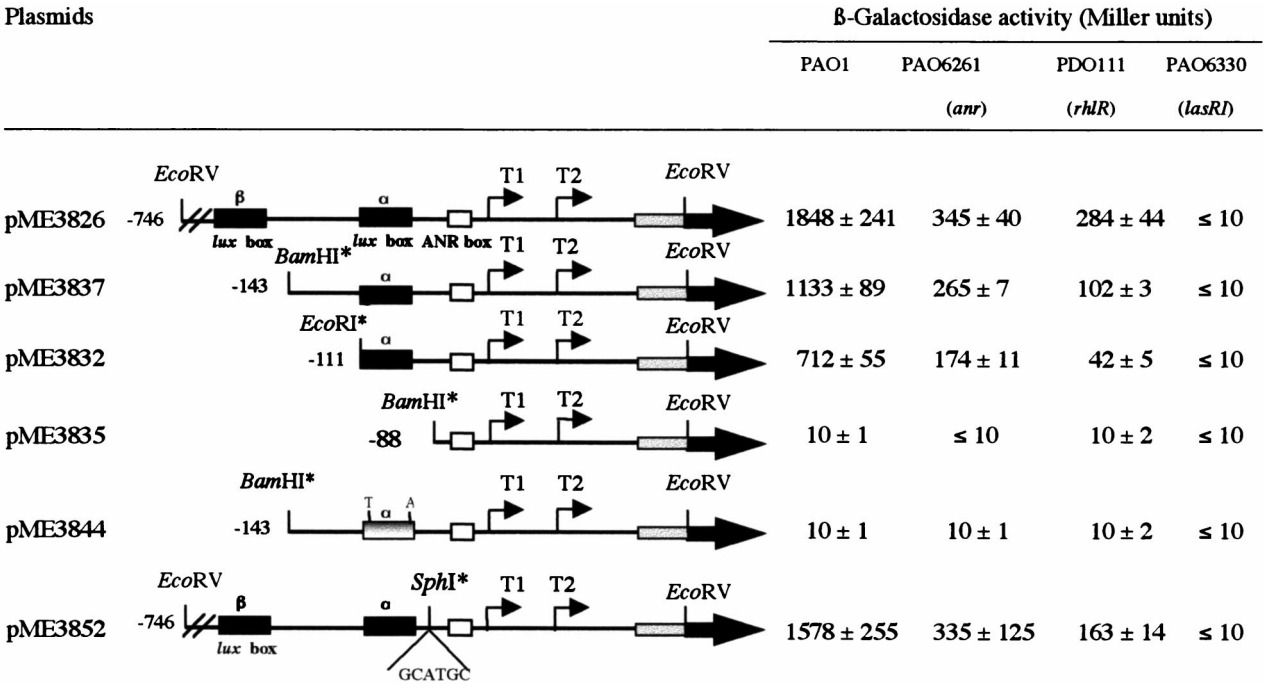

FIG. 6. Effects of deletions and site-directed mutations on *hcnA* promoter activity. β-Galactosidase activities were determined in *P. aeruginosa* wild type PAO1 and in mutants PDO111 (*rhlR*), PAO6330 (*lasRI*), and PAO6261 (*anr*) containing the *hcnA′-′lacZ* fusion plasmids shown. Cells were grown in 40 ml of MMC with mild oxygen limitation to an OD_{600} of 1.0 to 1.3. Restriction sites introduced artificially are indicated by ∗. The mutated *lux* box α of pME3844 is indicated by two nucleotides mutated (T, A), and the *hcnA* gene is shown in gray. β-Galactosidase activity is provided along with the standard deviation (mean of three independent experiments).

(and *N*-hexanoyl-homoserine lactone) by strain CHA0 containing pME3837 and pME3840 was verified by a bioassay (see Materials and Methods); in the vector control (CHA0 harboring pME6001 instead of pME3840), no BHL was detected. Introduction of the *P. aeruginosa lasR* gene on pME3827 into *P. fluorescens* CHA0/pME3837 also enhanced *hcnA′-′lacZ* expression fourfold, provided that 50 nM OdDHL was added to the culture medium (Table 4). When the crucial *lux* box α was mutated at two positions (pME3844 [Fig. 6]), *hcnA′-′lacZ* expression was stimulated by neither LasR/OdDHL nor RhlR/BHL in *P. fluorescens* (Table 4). Attempts to introduce both LasR and RhlR into *P. fluorescens* were unsuccessful. Nevertheless, the data presented in Table 4 show that LasR/OdDHL alone or RhlR/BHL alone can activate the *hcn* promoter to some extent.

DISCUSSION

Early physiological experiments on HCN formation in *P. aeruginosa*, which showed that both the growth phase and reduced oxygen levels are important for induction (9, 11, 12), can now be explained in the light of this study on the *hcn* promoter. With increasing cell density, expression of the quorum-sensing regulators LasR and RhlR increases as does the concentration of OdDHL and BHL (32, 44, 45). As a consequence, transcription from start site T1 is enhanced in parallel (Fig. 4), presumably because both LasR/OdDHL and RhlR/BHL can bind to *lux* box α. Although each regulator alone can achieve partial activation of this promoter (Table 4), the combined action of both regulators is more effective (Table 3); for this reason, our model (Fig. 7) pictures a hypothetical RhlR-LasR heterodimer. The RhlR-LasR order shown is arbitrary; the opposite order could also be true. Expression of elastase in *P. aeruginosa* shows a similar dependence on LasR and RhlR, whereas the expression of rhamnolipid biosynthetic genes is regulated tightly by RhlR alone (7). In the *hcn* promoter, *lux* box α is centered at −42.5 bp relative to the transcription start T1. As noted previously for LuxR in *V. fischeri*, a 42.5-bp spacing between the center of the *lux* box and the transcription

TABLE 3. Effects of added autoinducers OdDHL and BHL on a translational *hcnA′-′lacZ* fusion (pME3823) in *P. aeruginosa* wild type and regulatory mutants[a]

Strain (genotype)	β-Galactosidase activity (Miller units)			
	No addition	+BHL	+OdDHL	+BHL +OdDHL
PAO1/pME3823 (wild type)	3,022 ± 112	ND	ND	ND
PAOJP2/pME3823 (*rhlI lasI*)	24 ± 6	400 ± 13	455 ± 4	2,197 ± 22
PAO6351/pME3823 (*lasI rhlR*)	112 ± 1	123 ± 8	581 ± 70	766 ± 76

[a] The activity of an *hcnA′-′lacZ* translational fusion construct (pME3823) was determined when cells grown in 40 ml of MMC (in 100-ml Erlenmeyer flasks) at 37°C with shaking reached an OD_{600} of 1.0 to 1.2. BHL was dissolved in 15% acetonitrile and added at a concentration of 10 μM; OdDHL was dissolved in 50% acetonitrile and added at a concentration of 10 μM. β-Galactosidase activities were determined in three independent experiments; means ± standard deviations are given. ND, not determined.

TABLE 4. LasR- and RhlR-dependent expression of the *hcnA* promoter of *P. aeruginosa* in *P. fluorescens* CHA0[a]

Plasmids	Regulator present	β-Galactosidase activity (Miller units)		
		No addition	+BHL	+OdDHL
pME3837 + pME6001		103 ± 17	ND	ND
pME3837 + pME3840	RhlRI	421 ± 32	452 ± 36	ND
pME3837 + pME3827	LasR	94 ± 5	ND	441 ± 36
pME3844 + pME3827	LasR	62 ± 3	ND	89 ± 7
pME3844 + pME3840	RhlRI	60 ± 6	69 ± 4	68 ± 5

[a] The *hcnA'-'lacZ* fusion constructs pME3837 and pME3844 (Fig. 6) were tested in strain CHA0. Cells were grown in 40 ml of MMC (in 100-ml Erlenmeyer flask) at 30°C with shaking to an OD_{600} of 1.0 to 1.2. BHL was dissolved in 15% acetonitrile and added at a concentration of 10 μM; OdDHL was dissolved in 50% acetonitrile and added at a concentration of 50 nM. β-Galactosidase activities were determined in triplicate; means ± standard deviations are given. ND, not determined.

start site (17) is characteristic of ambidextrous transcriptional activators, such as CRP (cyclic AMP receptor protein) and FNR, which interact with the C-terminal domain of the α subunits of RNA polymerase and with σ^{70} (4, 46).

At low oxygen levels, the ANR protein provides additional *hcn* expression, by activating transcription from start site T2 (Fig. 4 and 6). The ANR protein itself appears to be expressed constitutively in *P. aeruginosa* (50). Interestingly, the function of promoter P2 was strictly dependent on the quorum-sensing machinery. As shown by pME3835, which lacks *lux* box α, and pME3844, which has a mutant variant of this *lux* box, ANR alone does not promote *hcn* transcription under mild oxygen limitation (Fig. 6). Even under severe oxygen limitation, no significant expression was observed with these constructs (data not shown). The reason for ANR not acting as an independent activator probably lies in the poorly conserved left half-site of the ANR box. The tandem arrangement of the *lux* and ANR boxes spaced 29 bp apart (Fig. 7) allows synergistic promoter activation by LasR, RhlR, and ANR (Fig. 6; Table 2). An analogous situation has been studied extensively by Belyaeva et al., who showed that *E. coli* promoters carrying tandem recognition sites for CRP at −41.5 and −71.5 bp are synergistically activated by CRP (4). The spacing between the sites can be increased with relatively minor consequences for promoter strength, suggesting a remarkable flexibility of the interactions between CRP and the subunits of RNA polymerase. It is therefore not surprising that a 6-bp insertion between the *lux* box and the ANR box affected *hcn* expression very little (Fig. 6). Because CRP, FNR, ANR, LuxR, LasR, and RhlR all have similar spacing requirements for their recognition sites, we have modeled the *hcn* promoter P2 (Fig. 7) after the tandem CRP promoter (−74.5/−41.5) (4). Binding of ANR to the *hcn* promoter not only activates transcription from T2 but also represses that from T1 (Fig. 6). The promoter region upstream of *lux* box α enhances *hcn* expression about threefold (Fig. 6). This region contains a second, suboptimal *lux* box (β) (Fig. 2B and 5), which could account for part of the enhancing effect. Although we did not investigate this in detail, we wish to point out the resemblance to the *lasB* promoter of *P. aeruginosa*, where two LasR recognition sites lying 60 bp apart synergistically enhance *lasB* promoter strength (1).

In *P. aeruginosa*, ANR is known to control anaerobic respiration with nitrate or nitrite as the electron acceptor (2) and the anaerobically inducible arginine deiminase pathway encoded by the *arcDABC* operon (62). It is interesting to compare the role of ANR as an activator of the *arc* and *hcn* promoters. At the *arc* promoter, ANR and the ANR box (located at −41.5 bp from the transcriptional start site) are sufficient to give strong anaerobic activation (25, 58). In the presence of arginine, the ArgR regulatory protein boosts the expression of the *arc* promoter by binding to the −70 region (35). Without ANR, ArgR does not activate this promoter (35). At the *hcn* promoter, by contrast, it is ANR that has an auxiliary role and the regulators (LasR and RhlR) binding to the upstream region can function autonomously.

In *P. fluorescens* CHA0, cyanogenesis also depends on ANR (34) and on cell density (6). However, in this organism the *hcn* promoter does not contain any *lux* box, and deletion of the region upstream of the ANR box does not affect *hcn* expression (5a). Furthermore, *P. fluorescens* CHA0 does not appear to produce *N*-acyl-homoserine lactones, although heterologous expression of the *P. aeruginosa rhlI* gene results in the synthesis of BHL by this strain (Table 4). A posttranscriptional mechanism of quorum-sensing regulation has been proposed for *P. fluorescens* CHA0 (6). Whether such a mechanism is conserved in *P. aeruginosa* remains to be seen. We find it striking that HCN production in one fluorescent pseudomonad, *P. aeruginosa* PAO, depends on the complex interactions between the *las* and the *rhl* systems, whereas in a closely related species, *P. fluorescens* CHA0, cyanogenesis can do without.

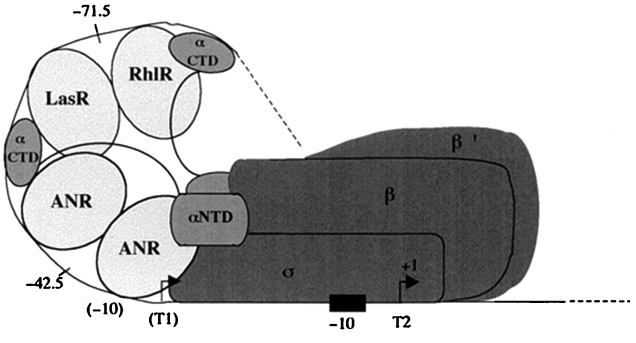

FIG. 7. Model for the recognition of the *hcn* promoter P2 by the transcriptional regulators ANR, LasR, and RhlR and their interaction with RNA polymerase. αCTD (C-terminal domain of α) and αNTD (N-terminal domain of α) of RNA polymerase are shown as separate domains joined by a linker. Positions relative to the transcriptional start site T2 are indicated below the sequence. This model has been adapted from that proposed by Belyaeva et al. (4) for CRP-dependent promoter activation. During oxygen limitation, ANR blocks transcription from site T1 by binding to the corresponding −10 sequence (in brackets).

ACKNOWLEDGMENTS

We thank Paul Williams for generously supplying autoinducer samples, and we thank B. Iglewski, J. Pearson, D. Ohman, C. Reimmann, and A. Lazdunski for providing strains. We also thank Steve Busby, Birgit Wackwitz, Karin Heurlier, Caroline Blumer, and Laura Serino for discussion and SmithKline Beecham for generously supplying carbenicillin.

This work was supported by the Swiss National Foundation for Scientific Research (31-56608.99) and European Biotechnology project BIO4CT960119.

REFERENCES

1. **Anderson, R. M., C. A. Zimprich, and L. Rust.** 1999. A second operator is involved in *Pseudomonas aeruginosa* elastase (*lasB*) activation. J. Bacteriol. **181:**6264–6270.
2. **Arai, H., T. Kodama, and Y. Igarashi.** 1997. Cascade regulation of the two CRP/FNR-related transcriptional regulators (ANR and DNR) and the denitrification enzymes in *Pseudomonas aeruginosa*. Mol. Microbiol. **25:**1141–1148.
3. **Bagdasarian, M. M., E. Amann, R. Lurz, B. Rückert, and M. Bagdasarian.** 1983. Activity of the hybrid *trp-lac* (*tac*) promoter of *Escherichia coli* in

Pseudomonas putida. Construction of broad-host-range, controlled-expression vectors. Gene **26:**273–282.
4. **Belyaeva, T. A., V. A. Rhodius, C. L. Webster, and S. J. Busby.** 1998. Transcription activation at promoters carrying tandem DNA sites for the *Escherichia coli* cyclic AMP receptor protein: organisation of the RNA polymerase alpha subunits. J. Mol. Biol. **277:**789–804.
5. **Blumer, C., and D. Haas.** 2000. Mechanism, regulation, and ecological role of bacterial cyanide biosynthesis. Arch. Microbiol. **173:**170–177.
5a. **Blumer, C., and D. Haas.** 2000. 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. Microbiology **146:**2417–2424.
6. **Blumer, C., S. Heeb, G. Pessi, and D. Haas.** 1999. Global GacA-steered control of cyanide and exoprotease production in *Pseudomonas fluorescens* involves specific ribosome binding sites. Proc. Natl. Acad. Sci. USA **96:** 14073–14078.
7. **Brint, J. M., and D. E. Ohman.** 1995. Synthesis of multiple exoproducts in *Pseudomonas aeruginosa* is under the control of RhlR-RhlI, another set of regulators in strain PAO1 with homology to the autoinducer-responsive LuxR-LuxI family. J. Bacteriol. **177:**7155–7163.
8. **Castric, P. A.** 1977. Glycine metabolism by *Pseudomonas aeruginosa*: hydrogen cyanide biosynthesis. J. Bacteriol. **130:**826–831.
9. **Castric, P. A.** 1983. Hydrogen cyanide production by *Pseudomonas aeruginosa* at reduced oxygen levels. Can. J. Microbiol. **29:**1344–1349.
10. **Castric, P. A.** 1975. Hydrogen cyanide, a secondary metabolite of *Pseudomonas aeruginosa*. Can. J. Microbiol. **21:**613–618.
11. **Castric, P. A.** 1994. Influence of oxygen on the *Pseudomonas aeruginosa* hydrogen cyanide synthase. Curr. Microbiol. **29:**19–21.
12. **Castric, P. A., R. F. Ebert, and K. F. Castric.** 1979. The relationship between growth phase and cyanogenesis in *Pseudomonas aeruginosa*. Curr. Microbiol. **2:**287–292.
13. **Cha, C., P. Gao, Y. C. Chen, P. D. Shaw, and S. K. Farrand.** 1998. Production of acyl-homoserine lactone quorum-sensing signals by gram-negative plant-associated bacteria. Mol. Plant-Microbe Interact. **11:**1119–1129.
14. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
15. **Del Sal, G., G. Manfioletti, and C. Schneider.** 1988. A one-tube plasmid DNA mini-preparation suitable for sequencing. Nucleic Acids Res. **16:**9878.
16. **Döring, G.** 1993. *Pseudomonas aeruginosa* infection in cystic fibrosis patients, p. 245–273. *In* M. Campa, M. Bendinelli, and H. Friedman (ed.), *Pseudomonas aeruginosa* as an opportunistic pathogen. Plenum Press, New York, N.Y.
17. **Egland, K. A., and E. P. Greenberg.** 1999. Quorum sensing in *Vibrio fischeri*: elements of the *luxI* promoter. Mol. Microbiol. **31:**1197–1204.
18. **Farinha, M. A., and A. M. Kropinski.** 1990. High efficiency electroporation of *Pseudomonas aeruginosa* using frozen cell suspensions. FEMS Microbiol. Lett. **58:**221–225.
19. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc. Natl. Acad. Sci. USA **76:**1648–1652.
20. **Fuqua, C., and E. P. Greenberg.** 1998. Self perception in bacteria: quorum sensing with acylated homoserine lactones. Curr. Opin. Microbiol. **1:**183–189.
21. **Fuqua, C., S. C. Winans, and E. P. Greenberg.** 1996. Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators. Annu. Rev. Microbiol. **50:**727–751.
22. **Fürste, J. P., W. Pansegrau, R. Frank, H. Blöcker, P. Scholz, M. Bagdasarian, and E. Lanka.** 1986. Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tac*P expression vector. Gene **48:**119–131.
23. **Gambello, M. J., and B. H. Iglewski.** 1991. Cloning and characterization of the *Pseudomonas aeruginosa lasR* gene, a transcriptional activator of elastase expression. J. Bacteriol. **173:**3000–3009.
24. **Gamper, M., B. Ganter, M. R. Polito, and D. Haas.** 1992. RNA processing modulates the expression of the *arcDABC* operon in *Pseudomonas aeruginosa*. J. Mol. Biol. **226:**943–957.
25. **Gamper, M., A. Zimmerman, and D. Haas.** 1991. Anaerobic regulation of transcription initiation in the *arcDABC* operon of *Pseudomonas aeruginosa*. J. Bacteriol. **173:**4742–4750.
26. **Gewitz, H. S., E. K. Pistorius, H. H. Voss, and B. Vennesland.** 1976. Cyanide formation in preparations from *Chlorella vulgaris* Beijerinck: effect of sonication and amygdalin addition. Planta **131:**145–148.
27. **Goldfarb, W. B., and H. Margraf.** 1967. Cyanide production by *Pseudomonas aeruginosa*. Ann. Surg. **165:**104–110.
28. **Heeb, S., Y. Itoh, T. Nishijyo, U. Schnider, C. Keel, J. Wade, U. Walsh, F. O'Gara, and D. Haas.** 2000. Small, stable shuttle vectors based on the minimal pVS1 replicon for use in gram-negative, plant-associated bacteria. Mol. Plant-Microbe Interact. **13:**232–237.
29. **Holder, I. A.** 1993. *Pseudomonas aeruginosa* burn infections: pathogenesis and treatment, p. 275–295. *In* M. Campa, M. Bendinelli, and H. Friedman (ed.), *Pseudomonas aeruginosa* as an opportunistic pathogen. Plenum Press, New York, N.Y.
30. **Holloway, B. W.** 1969. Genetics of *Pseudomonas*. Bacteriol. Rev. **33:**419–443.
31. **Kovach, M. E., R. W. Phillips, P. H. Elzer, R. M. Roop II, and K. M. Peterson.** 1994. pBBR1MCS: a broad-host-range cloning vector. BioTechniques **16:**800–802.
32. **Latifi, A., M. Foglino, K. Tanaka, P. Williams, and A. Lazdunski.** 1996. A hierarchical quorum-sensing cascade in *Pseudomonas aeruginosa* links the transcriptional activators LasR and RhlR (VsmR) to expression of the stationary-phase sigma factor RpoS. Mol. Microbiol. **21:**1137–1146.
33. **Latifi, A., M. K. Winson, M. Foglino, B. W. Bycroft, G. S. Stewart, A. Lazdunski, and P. Williams.** 1995. Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in *Pseudomonas aeruginosa* PAO1. Mol. Microbiol. **17:**333–343.
34. **Laville, J., C. Blumer, C. Von Schroetter, V. Gaia, G. Défago, C. Keel, and D. Haas.** 1998. Characterization of the *hcnABC* gene cluster encoding hydrogen cyanide synthase and anaerobic regulation by ANR in the strictly aerobic biocontrol agent *Pseudomonas fluorescens* CHA0. J. Bacteriol. **180:** 3187–3196.
35. **Lu, C. D., H. Winteler, A. Abdelal, and D. Haas.** 1999. The ArgR regulatory protein, a helper to the anaerobic regulator ANR during transcriptional activation of the *arcD* promoter in *Pseudomonas aeruginosa*. J. Bacteriol. **181:**2459–2464.
36. **Matsumoto, H., S. Ohta, R. Kobayashi, and Y. Terawaki.** 1978. Chromosomal location of genes participating in the degradation of purines in *Pseudomonas aeruginosa*. Mol. Gen. Genet. **167:**165–176.
37. **Maurhofer, M., C. Reimmann, P. Schmidli-Sacherer, S. Heeb, D. Haas, and G. Défago.** 1998. Salicylic acid biosynthetic genes expressed in *Pseudomonas fluorescens* strain P3 improve the induction of systemic resistance in tobacco against tobacco necrosis virus. Phytopathology **88:**678–684.
38. **McClean, K. H., M. K. Winson, L. Fish, A. Taylor, S. R. Chhabra, M. Camara, M. Daykin, J. H. Lamb, S. Swift, B. W. Bycroft, G. S. Stewart, and P. Williams.** 1997. Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. Microbiology **143:**3703–3711.
39. **Mikaelian, I., and A. Sergeant.** 1996. Modification of the overlap extension method for extensive mutagenesis on the same template. Methods Mol. Biol. **57:**193–202.
40. **Minton, N. P.** 1984. Improved plasmid vectors for the isolation of translational *lac* gene fusions. Gene **31:**269–273.
41. **Morgan, A. F.** 1979. Transduction of *Pseudomonas aeruginosa* with a mutant of bacteriophage E79. J. Bacteriol. **139:**137–140.
42. **Ochsner, U. A., A. K. Koch, A. Fiechter, and J. Reiser.** 1994. Isolation and characterization of a regulatory gene affecting rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. J. Bacteriol. **176:**2044–2054.
43. **Pearson, J. P., E. C. Pesci, and B. H. Iglewski.** 1997. Roles of *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. J. Bacteriol. **179:**5756–5767.
44. **Pesci, E. C., J. P. Pearson, P. C. Seed, and B. H. Iglewski.** 1997. Regulation of *las* and *rhl* quorum sensing in *Pseudomonas aeruginosa*. J. Bacteriol. **179:**3127–3132.
45. **Reimmann, C., M. Beyeler, A. Latifi, H. Winteler, M. Foglino, A. Lazdunski, and D. Haas.** 1997. The global activator GacA of *Pseudomonas aeruginosa* PAO positively controls the production of the autoinducer *N*-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide, and lipase. Mol. Microbiol. **24:**309–319.
46. **Rhodius, V. A., and S. J. W. Busby.** 1998. Positive activation of gene expression. Curr. Opin. Microbiol. **1:**152–159.
47. **Rumbaugh, K. P., J. A. Griswold, B. H. Iglewski, and A. N. Hamood.** 1999. Contribution of quorum sensing to the virulence of *Pseudomonas aeruginosa* in burn wound infections. Infect. Immun. **67:**5854–5862.
48. **Rust, L., E. C. Pesci, and B. H. Iglewski.** 1996. Analysis of the *Pseudomonas aeruginosa* elastase (*lasB*) regulatory region. J. Bacteriol. **178:**1134–1140.
49. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, N.Y.
50. **Savioz, A., A. Zimmermann, and D. Haas.** 1993. *Pseudomonas aeruginosa* promoters which contain a conserved GG-N10-GC motif but appear to be RpoN-independent. Mol. Gen. Genet. **238:**74–80.
51. **Seed, P. C., L. Passador, and B. H. Iglewski.** 1995. Activation of the *Pseudomonas aeruginosa lasI* gene by LasR and the *Pseudomonas* autoinducer PAI: an autoinduction regulatory hierarchy. J. Bacteriol. **177:**654–659.
52. **Solomonson, L. P.** 1981. Cyanide as a metabolic inhibitor, p. 11–28. *In* B. Vennesland, E. E. Conn, C. J. Knowles, J. Westley, and F. Wissing (ed.), Cyanide in biology. Academic Press Ltd., London, England.
53. **Spiro, S.** 1994. The FNR family of transcriptional regulators. Antonie Leeuwenhoek **66:**23–36.
54. **Vögtli, M., and R. Hütter.** 1987. Characterisation of the hydroxystreptomycin phosphotransferase gene (*sph*) of *Streptomyces glaucescens*: nucleotide sequence and promoter analysis. Mol. Gen. Genet. **208:**195–203.
55. **Voisard, C., C. T. Bull, C. Keel, J. Laville, M. Maurhofer, U. Schnider, G. Défago, and D. Haas.** 1994. Biocontrol of root diseases by *Pseudomonas fluorescens* CHA0: current concepts and experimental approaches, p. 67–89. *In* F. O'Gara, D. N. Dowling, and B. Boesten (ed.), Molecular ecology of

rhizosphere microorganisms. VCH, Weinheim, Germany.
56. **Whiteley, M., K. M. Lee, and E. P. Greenberg.** 1999. Identification of genes controlled by quorum sensing in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **96:**13904–13909.
57. **Winson, M. K., M. Camara, A. Latifi, M. Foglino, S. R. Chhabra, M. Daykin, M. Bally, V. Chapon, G. P. Salmond, B. W. Bycroft, A. Lazdunski, G. S. A. B. Stewart, and P. Williams.** 1995. Multiple *N*-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **92:**9427–9431.
58. **Winteler, H. V., and D. Haas.** 1996. The homologous regulators ANR of *Pseudomonas aeruginosa* and FNR of *Escherichia coli* have overlapping but distinct specificities for anaerobically inducible promoters. Microbiology **142:** 685–693.
59. **Winteler, H. V., B. Schneidinger, K. E. Jaeger, and D. Haas.** 1996. Anaerobically controlled expression system derived from the *arcDABC* operon of *Pseudomonas aeruginosa*: application to lipase production. Appl. Environ. Microbiol. **62:**3391–3398.
60. **Wissing, F., and K. A. Andersen.** 1981. The enzymology of cyanide production from glycine by a *Pseudomonas* species, p. 275–288. *In* B. Vennesland, E. E. Conn, C. J. Knowles, J. Westley, and F. Wissing (ed.), Cyanide in biology. Academic Press Ltd., London, England.
61. **Ye, R. W., D. Haas, J. O. Ka, V. Krishnapillai, A. Zimmermann, C. Baird, and J. M. Tiedje.** 1995. Anaerobic activation of the entire denitrification pathway in *Pseudomonas aeruginosa* requires Anr, an analog of Fnr. J. Bacteriol. **177:**3606–3609.
62. **Zimmerman, A., C. Reimmann, M. Galimand, and D. Haas.** 1991. Anaerobic growth and cyanide synthesis of *Pseudomonas aeruginosa* depend on *anr*, a regulatory gene homologous with *fnr* of *Escherichia coli*. Mol. Microbiol. **5:**1483–1490.