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Anaerobic Regulation of Transcription Initiation in the *arcDABC* Operon of *Pseudomonas aeruginosa*

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The *arcDABC* operon of *Pseudomonas aeruginosa* encodes the enzymes of the arginine deiminase pathway, which is inducible under conditions of oxygen limitation and serves to generate ATP from arginine. The 5′ end of *arc* mRNA extracted from anaerobically grown cells was determined by S1 and primer extension mapping. The transcription initiation site was located upstream of the *arcD* gene and 41.5 bp downstream of the center of the sequence TTGAC....ATCAG. This sequence, termed the ANR box, is similar to the consensus FNR recognition site of *Escherichia coli*. Transcription of the *arc* operon in *P. aeruginosa* was strongly decreased by a deletion of the TTGAC half site or by a mutation in the *anr* gene, which is known to code for the FNR-like regulatory protein ANR. During a transition from aerobic to anaerobic growth conditions, the concentrations of *arc* mRNAs and the levels of the ArcD and ArcA proteins rose in a parallel fashion. Mutational analysis of the *arc* promoter region led to the conclusion that the distance between the ANR box and the −10 promoter region is important for promoter strength, whereas the −35 region does not appear to be critical for *arc* promoter function. These findings and previous results indicate that anaerobic induction of the *arc* operon occurs at the level of transcription and requires the ANR box in *cis* and the ANR protein in *trans*.

To obtain metabolic energy, *Pseudomonas aeruginosa* preferentially uses aerobic respiration. Under oxygen-limiting conditions, in the presence of nitrate or nitrite, *P. aeruginosa* switches to anaerobic respiration (9, 37). In the absence of terminal electron acceptors, arginine can be used as the sole energy source (52). Anaerobic degradation of arginine to ornithine produces 1 mol of ATP per mol of arginine and depends on the three enzymes of the arginine deiminase pathway: arginine deiminase (ADI), catabolic ornithine carbamoyltransferase, and carbamate kinase (Fig. 1). These enzymes are encoded by the *arcA*, *arcB*, and *arcC* genes, respectively (29), and are induced coordinately when the dissolved oxygen tension falls below 1% air saturation (32). An additional gene, *arcD*, codes for an arginine-ornithine antiporter located in the cytoplasmic membrane (6). Nucleotide sequence analysis and transcriptional fusions indicate that the *arc* genes are oriented in the same direction within a 5-kb DNA segment, forming the *arcDABC* operon (2, 3, 28, 29). Deletions extending from the region upstream of *arcD* into the proximal part of *arcD* abolish almost completely the inducible synthesis of the *arcABC* enzymes. Similarly, insertions of IS*21* or Tn*1725* in the *arcD* coding sequence have a strong polar effect on the expression of the downstream *arcABC* genes (28). These results suggest that the anaerobically regulated promoter of the *arc* operon is located upstream of the *arcD* gene.

Under low oxygen tension, expression of the ADI pathway in *P. aeruginosa* depends on the *anr* gene, which specifies the *trans*-acting positive regulator ANR (18). Mutations in the *anr* gene pleiotropically block the induction of the ADI pathway and of nitrate and nitrite reductases. Therefore, *anr* mutants cannot grow anaerobically on arginine or nitrate (56). The cloned *anr* gene of *P. aeruginosa* functionally complements an *fnr* mutant of *Escherichia coli* for anaerobic growth on nitrate medium. ANR and FNR have 51% sequence identity, and several amino acid residues known to be essential for FNR function are strictly conserved in ANR (18, 26, 31, 46, 56). Thus, FNR and ANR appear to have similar functions.

In anaerobically growing enteric bacteria, FNR recognizes specific conserved sequences whose consensus is TTGAT....ATCAA (12, 46). In FNR-dependent promoters this sequence occurs about 40 bp upstream of the transcription initiation site, enabling FNR to function as a transcriptional activator. Some other promoters, including that of the *fnr* gene itself, contain the FNR recognition sequence around the transcription start point. At these promoters, FNR acts as a repressor under anaerobic conditions (12, 46, 47).

We previously showed that a sequence resembling the consensus FNR recognition sequence is located upstream of the *arcDABC* operon of *P. aeruginosa* and that this sequence is important for the anaerobic expression of ADI (18). Here we show that this sequence (termed the ANR box) is required in *cis* for ANR-dependent transcription of the *arc* operon, and we characterize the *arc* promoter.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used are listed in Table 1. For cloning experiments, *E. coli* RR28 was used.

Media and growth conditions. The media for aerobic growth (nutrient agar, nutrient yeast broth, minimal medium E [40, 48]) and anaerobic growth (yeast extract-arginine [YEA] [52]) of *P. aeruginosa* have been described. All incubations were carried out at 37°C. The selective antibiotic concentrations used were those of Rella et al. (40), except that for maintenance of pKT240 derivatives carbenicillin was used at 300 μg/ml in strains PAO1 and PTO6235 and at 100 μg/ml in strain PAO6251.

DNA manipulations. The procedures of Del Sal et al. (10) and Itoh et al. (25) were used for small- and large-scale preparations, respectively, of plasmid DNA. Restriction analysis, agarose gel electrophoresis, DNA fragment isola-

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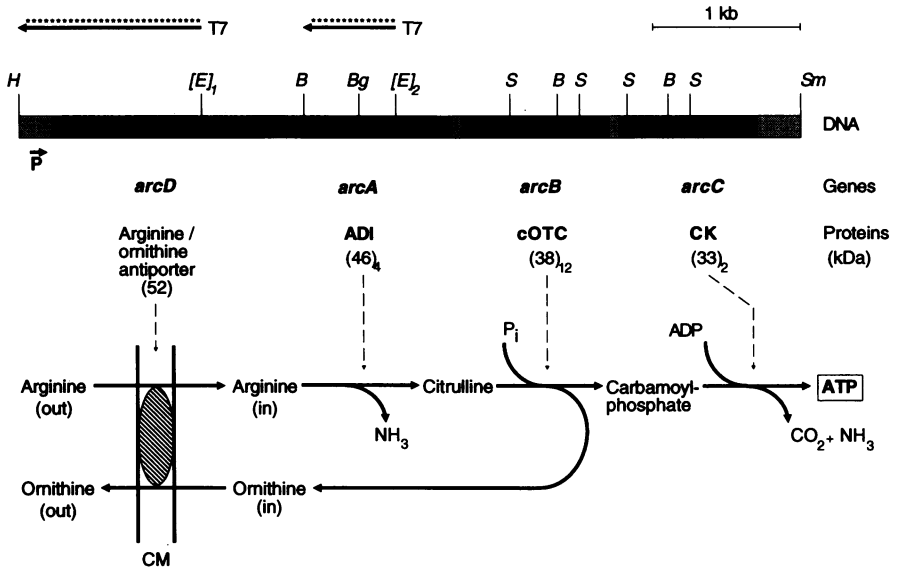


FIG. 1. The *arc* operon (5.3 kb) and the arginine deiminase pathway of *P. aeruginosa*. P, promoter of the *arc* operon; CM, cytoplasmic membrane; cOTC, catabolic ornithine carbamoyltransferase; CK, carbamate kinase. Restriction sites: *H*, *Hin*dIII; *B*, *Bam*HI; *Bg*, *Bgl*II; *S*, *Sac*II; *Sm*, *Sma*I; *E*, *Eco*RI. The *Hin*dIII site is defined as position 1 (base pair) or 0 kb in the *arc* operon. E_1 and E_2 do not exist in the wild-type *arc* operon. They were derived from Tn*1725* insertions (50) and subsequent excision of Tn*1725* with *Eco*RI (28). E_1 is at position 1241 from *Hin*dIII, E_2 is situated at position ca. 2590 (28). The two arrows labeled with asterisks indicate the location and direction of the antisense RNA probes synthesized in vitro with T7 RNA polymerase and pME3701 or pME3702 as the template (30). These probes were used to monitor mRNA levels in the *arcD* and *arcA* gene regions (see Fig. 4 and 5).

tion from low-melting-point agarose gels, ligations, filling-in reactions with Klenow DNA polymerase, trimming of 3′ protruding ends with T4 DNA polymerase, dephosphorylation with alkaline phosphatase, end labeling with polynucleotide kinase, and $CaCl_2$-dependent transformation were all performed by standard methods (41). For DNA sequencing, M13mp19 (33) and pBluescript (Stratagene) vectors served to generate single-stranded DNA in strain TG1; sequencing reactions were carried out with the Klenow enzyme as described by Baur et al. (3) or with Sequenase (U.S. Biochemical Corp., Cleveland, Ohio). For oligonucleotide-directed in vitro mutagenesis (49), the Amersham protocol (version 2) was followed. The mutagenic primers 5′-ATC TCCCTATAGGAATTCTGATCCACGTCAATA-3′ and 5′-ATTAGGCGGCAGGCCTGCAGCGAATTCCCTGATC CACGTC-3′ were used to construct mutated *arc* promoters in pBluescript derivatives carrying a 0.65-kb *arc* promoter fragment. The mutated fragments were subcloned into pKT240 derivatives, giving pME3730 and pME3731, respectively (Table 1) (see Fig. 4). Recombinant plasmids derived from pKT240 were transferred to *P. aeruginosa* by mobilization with pRK2013 (18).

Construction of chromosomal *arc* mutants of *P. aeruginosa*. The *arcA* gene was inactivated by insertion of the transcription-translation stop element Ω-Hg (14) as follows. A *Bam*HI fragment carrying the Ω-Hg element was cloned into the unique *Bgl*II site of the *arc* operon (Fig. 1) in the pBR325 derivative pME327 (Table 1). The origin of transfer (*oriT*) of the broad-host-range plasmid RP1 was then inserted into pME327::Ω-Hg by cointegration in vivo with the replication-deficient mini-RP1 plasmid pME28 (39). A pME327::Ω-Hg::pME28 cointegrate thus obtained contained the pME28 insertion in the pBR325 vector moiety, i.e., outside the *arc* operon. The cointegrate was then introduced into *E. coli* S17-1, which carries the RP4 (RP1) transfer genes in the chromosome (43) and mobilizes pME28 derivatives at high frequencies (39). The S17-1 (pME327::Ω-Hg::pME28) donor was mated with *P. aeruginosa* PAO1, with delayed selection for Hg resistance on minimal medium containing 25 μg of $HgCl_2$ per ml. Colonies were formed at 10^{-8} per donor. Since the ColE1 replication system of the cointegrate does not function in *P. aeruginosa*, Hg-resistant colonies are formed as a result of recombination between the cointegrate and the chromosome. A single crossover is expected to integrate the entire cointegrate (observed in 62% of Hg-resistant colonies), whereas two crossovers in the sequences flanking the Ω-Hg cassette should result in a site-directed mutation of the chromosomal *arcA* gene (observed in 38% of Hg-resistant colonies). One representative strain of the latter class, PAO6235 (*arcA*::*mer*), was kept. Genomic DNA from this strain was digested with *Sal*I and subjected to Southern hybridization with *arc* operon DNA as a probe. The bands observed were consistent with an Ω-Hg insertion in *arcA* (data not shown). A *recA102* derivative, PTO6235, was then isolated in two steps. First, the *thr-6093*::Tn*5-751* marker (40) was introduced by E79*tv-2*-mediated transduction (36), with selection for transposon Tn*5-751*. Second, the PAO6235 Thr^- transductant was rendered Thr^+ in a mating with the *recA* donor PTO66(R68.45). Since *recA* is linked to *thr-6093* in R68.45-mediated conjugation (16, 40), it was possible to obtain Rec^- recombinants. Strain PTO6235 (*arcA*::*mer recA102*), which did not inherit the mobilizing plasmid R68.45, was retained.

To delete the entire chromosomal *arcDABC* operon, we used a similar strategy, but with pACYC184 as a mobilizable suicide vector (8). The construction of a pACYC184Δ*arc* plasmid can be summarized as follows. First, a 4.3-kb *Sma*I-*Bgl*II fragment, which lies downstream of the *arc* operon, was isolated from pME163 (29), and the *Sma*I site (shown in Fig. 1 at 5.3 kb) was replaced by an *Eco*RI linker. Second, a 3.2-kb *Bgl*II-*Hin*dIII fragment, which is located upstream of the *arc* operon, was taken from pME180 (29).

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or description	Reference or source
P. aeruginosa		
PAO1	Wild type	23
PAO6235	*arcA*::*mer*	This study
PAO6251	Δ*arcDABC*	This study
PTO66	*his-4 lysA12 ilv-1118 trp-6 proA82 recA102*, R68.45$^+$	16
PTO6235	*arcA*::*mer recA102*	This study
S1239	*ilvB112 met-9 leu-1 str anr* (*nirD*)	18, 53
E. coli		
RR28	*pheS12 hsdS20 recA13 proA2 leu-6 thi-1 ara-14 galK2 lacY1 xyl-5 mtl-1 supE44 rpsL20 endA*	22
S17-1	*pro thi hsdR recA*, chromosomal insertion of RP4.2	43
TG1	*hsdΔ5 thi supE* Δ(*lac-proAB*), F′ (*traD36 proA*$^+$*B*$^+$ *lacI*^{q} ZΔM15)	41
Plasmids		
pACYC184	Tc^r Cm^r P15A replicon (4.2 kb)	7
pBluescript KS (+)	Ap^r/Cb^r ColE1 replicon (3.0 kb)	42, Stratagene
pBR322	Ap^r/Cb^r Tc^r ColE1 replicon (4.4 kb)	5
pGEM-1	Ap^r/Cb^r ColE1 replicon (3.0 kb)	Promega
pHP45Ω-Hg	Ap^r/Cb^r Hg^r ColE1 replicon (7.1 kb)	14
pKT240	Ap^r/Cb^r Km^r Mob IncQ (12.5 kb)	1
pME28	Tc^r Km^r ΔTra2 (Tra$^-$) (IS*21*)$_2$ *trfA*::IS*21* (Rep$^-$) Mob IncP-1 (37.4 kb)	39
pME163	Ap^r/Cb^r Tp^r ColE1 replicon (ca. 29 kb), *arc′DABC* genes and the flanking downstream region (ca. 16 kb)	29
pME180	Ap^r/Cb^r ColE1 replicon (ca. 17 kb), *arcDABC* operon with flanking up- and downstream sequences	29
pME183	Ap^r/Cb^r Mob IncQ (17.5 kb); 5.5-kb *Hin*dIII-*Cla*I fragment carrying *arcDABC*, cloned into pKT240	29
pME190	Ap^r/Cb^r Mob IncQ (16.1 kb); 5.3-kb *Hin*dIII-*Sma*I fragment carrying *arcDABC*, cloned into pKT240	29
pME327	Ap^r/Cb^r Cm^r ColE1 replicon (11.5 kb), 5.5-kb *Hin*dIII-*Cla*I fragment carrying *arcDABC*	28
pME327::Ω-Hg	Ap^r/Cb^r Cm^r Hg^r ColE1 replicon (16.3 kb); 4.8-kb Ω-Hg fragment of pHP45Ω-Hg, cloned into *arcA* of pME327	This study
pME336	Ap^r/Cb^r Mob IncQ (15.6 kb), translational *arcD′-′lacZ* fusion, replacement of the 5.05-kb *Mae*I-*Sma*I fragment of pME183 (*arc′DABC*) by the 3.15-kb *Sma*I-*Aha*III *lacZ* fragment of pNM480	This study (Fig. 3)
pME340	Ap^r/Cb^r Mob IncQ (17.65 kb), translational *arcA′-′lacZ* fusion	56
pME3701	Ap^r/Cb^r ColE1 replicon (4.25 kb); 1.25-kb *Hin*dIII-*Eco*RI$_1$ fragment carrying *arc* promoter and *arcD* sequences, cloned into pGEM-1	This study (Fig. 1)
pME3702	Ap^r/Cb^r ColE1 replicon (3.65 kb); 0.65-kb *Bam*HI-*Eco*RI$_2$ fragment carrying an *arcA* internal sequence, cloned into pGEM-1	This study (Fig. 1)
pME3721-34	Ap^r/Cb^r Mob IncQ (14.0 kb); 5.2-kb *Hin*dIII-*Sma*I fragment carrying *arcDABC* with a deletion in the *arc* promoter sequence, cloned into pMMB67HE	This study (Fig. 3)
pME3722-23	Ap^r/Cb^r Mob IncQ (15.8 kb); 5.0-kb *Hin*dIII-*Sma*I fragment carrying *arc′DABC* without the *arc* promoter sequence and the *arcD N*-terminus, cloned into pKT240	This study
pME3722-34	Ap^r/Cb^r Mob IncQ (16.0 kb); 5.2-kb *Hin*dIII-*Sma*I fragment carrying *arcDABC* without the sequence upstream of the *arc* promoter, cloned into pKT240	This study (Fig. 3)
pME3730	Ap^r/Cb^r Mob IncQ (16.1 kb), deletion of 40 bp in the *arc* promoter region of pME190	This study (Fig. 3)
pME3731	Ap^r/Cb^r Mob IncQ (16.1 kb), substitution of 16 bp in the *arc* promoter region of pME190	This study (Fig. 3)
pME3735	Ap^r/Cb^r Mob IncQ (16.1 kb), insertion of 4 bp in the *arc* promoter region of pME3731	This study (Fig. 3)
pMMB67HE	Ap^r/Cb^r Mob IncQ (8.9 kb); expression vector with the *lacI*^{q} gene, the *tac* promoter, and a polylinker	17
pNM480	Ap^r/Cb^r ColE1 replicon (8.6 kb), *lacZ* translational fusion vector	35
pRK2013	Km^r Tra ColE1 replicon (ca. 50 kb)	15

The *Hin*dIII end (shown in Fig. 1 at 0 kb) of this fragment was fused, with a 12-bp linker, to the *Eco*RI end of the 4.3-kb downstream fragment. Thus, the entire *arc* operon between the *Hin*dIII and *Sma*I sites was deleted and exchanged for a short linker; this construct was then inserted into the chloramphenicol resistance determinant of pACYC184 cleaved with *Pvu*II. The mobilizing plasmid pRK2013, which is compatible with pACYC184, was introduced into *E. coli* RR28 carrying the pACYC184Δ*arc* construct. The latter plasmid was conjugationally mobilized to *P. aeruginosa* PAO1, with selection for tetracycline (50 μg/ml) resistance on minimal medium. This resulted in the integration of the

plasmid into the chromosome, at ca. 10^{-8} per donor. Enrichment for tetracycline-sensitive derivatives (in nutrient yeast broth containing 20 μg of tetracycline per ml and 4 mg of D-cycloserine per ml [20]) was then used to eliminate the integrated plasmid. Among several dozens of tetracycline-sensitive colonies, one (PAO6251) was found that was phenotypically Arc$^-$. We verified by Southern hybridization that the *arc* operon in strain PAO6251 was indeed deleted (data not shown). The low yield of Arc$^-$ colonies can perhaps be explained by the fact that PAO6251 grows poorly, even in aerobiosis. Thus, the enrichment procedure may have favored the recovery of wild-type clones.

RNA experiments. For dot blot hybridization experiments, *P. aeruginosa* strains were grown in 500-ml bottles filled with 500 ml of YEA medium and tightly closed with a rubber septum. The inoculum was 3.5 ml of an overnight, aerobic culture. At intervals, samples of 1.5 ml were withdrawn from the bottle with a syringe and RNA was isolated as described by Kullik et al. (27). Samples of total RNA (0.2 μg) were denatured (27) and applied to a Hybond-N membrane (Amersham) with a slot blotting apparatus (Schleicher & Schüll, Feldbach, Switzerland). Conditions for prehybridization, hybridization, and washes were according to the instructions supplied by Amersham and Kullik et al. (27). The radioactive probes used for hybridization consisted of [α-^{32}P]UTP-labeled *arcD* or *arcA* antisense RNA. They were synthesized in vitro with the pGEM-1-based plasmid pME3701 or pME3702 as the template, T7 RNA polymerase, and 40 μCi of [α-^{32}P]UTP in a reaction mix specified by Promega (Madison, Wis.). Plasmid pME3701 contains the 1.25-kb *Hin*dIII-*Eco*RI$_1$ fragment carrying *arcD'*, and pME3702 contains the 0.65-kb *Bam*HI-*Eco*RI$_2$ fragment carrying *'arcA'* (Fig. 1) behind the T7 promoter. Unincorporated nucleotides were separated from labeled RNA by Sephadex G-75 chromatography. Dot blot signals were quantitated by scanning with model 300A computing densitometer from Molecular Dynamics (Sunnyvale, Calif.).

For S1 and primer extension mapping, RNA was isolated as described by Hopwood et al. (24), with the following modifications. After oxygen-limited growth in 800 ml of YEA medium, the *P. aeruginosa* cells were harvested by centrifugation and broken in a Teflon tube containing 7 ml of Kirby mixture (24) and 14 g of glass beads. Phenol-chloroform extraction and digestion with DNase I (RNase free; Boehringer) were carried out as described by Hopwood et al. (24).

For S1 mapping, 100 μg of RNA was mixed with ^{32}P-labeled DNA (ca. 5×10^5 cpm; labeled with polynucleotide kinase at a *Bst*EII or *Sty*I site in *arcD* [Fig. 2a]) and incubated in 20 μl of hybridization solution (13) at 90°C for 10 min. The mixture was cooled to 62°C and hybridization was continued for 3 h. Then 300 μl of ice-cold S1 nuclease buffer (41) was added, and S1 nuclease digestion was performed at 37°C for 20 min. DNA protected from S1 was analyzed on a 4 or 6% polyacrylamide gel containing 7 M urea (41).

The conditions for primer extension mapping were as described by Vögtli and Hütter (54).

Enzyme assays. ADI was measured in toluenized cells as described by Lüthi et al. (29), and β-galactosidase assays were carried out as described by Miller et al. (34).

RESULTS

5' ends of *arc* transcripts synthesized under anaerobic conditions. To determine the site(s) of transcription initiation in the *arc* operon, we isolated total RNA from cultures of the wild-type strain PAO1 incubated under oxygen-limiting conditions for 6, 12, or 24 h. Low-resolution S1 mapping was carried out with an *arcD* probe labeled at the unique *Bst*EII site (position 845 relative to the *Hin*dIII site; Fig. 2a). End-labeled *Alu*I fragments of pBR322 and *Eco*RI-*Hin*dIII fragments of λ were used as size markers. In this experiment, a DNA fragment of about 650 nucleotides was protected from S1 nuclease and gave the predominant signal. The highest relative intensity of this signal was obtained with RNA isolated after 6 or 12 h (data not shown).

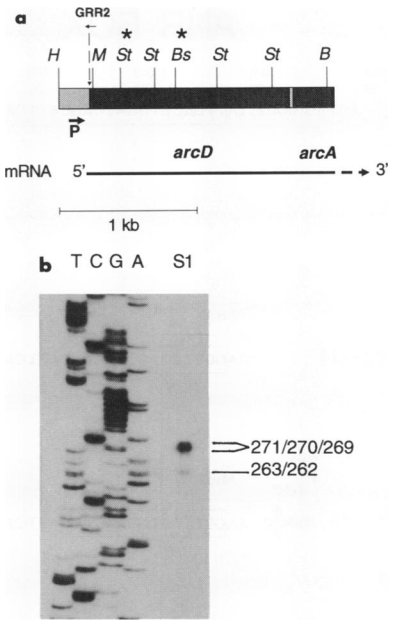


FIG. 2. (a) Proximal part of the *arc* operon. The direction of transcription in the *arc* operon, starting at the *arc* promoter (P), is indicated by an arrow (5' → 3'). For S1 mapping, the *Bst*EII (*Bs*) or *Sty*I (*St*) site was end labeled (*). The location of the oligonucleotide GRR2, which was used for primer extension analysis, is also shown. Further restriction sites are abbreviated as follows: *H*, *Hin*dIII; *M*, *Mae*I; *B*, *Bam*HI. (b) S1 mapping of the *arc* promoter. Total RNA isolated after 12 h of anaerobic growth was analyzed (lane S1). For hybridization, a DNA fragment uniquely labeled at the 5' end of the *Sty*I site (position 462 bp from the *Hin*dIII site) was used. On the right are shown the numbers of nucleotides protected from S1 nuclease. The Sanger sequence ladder of M13mp19 initiated by primer no. 1 (Bio-Rad; no. 170-3410) served as a size marker in a 6% polyacrylamide gel.

For high-resolution S1 mapping, the *Sty*I site at a position 462 bp from the *Hin*dIII site (Fig. 2a) was end labeled, and an M13mp19 sequence ladder was used as a size standard. With RNA extracted after 12 h of oxygen-limited incubation, protected DNA fragments of about 270 nucleotides were obtained. An additional minor signal corresponding to 263 nucleotides and possibly representing a degradation product was also visible (Fig. 2b). This places the 5' end of the longest *arcD* transcript at position 193 ± 1 (Fig. 3).

Primer extension analysis was performed with RNA isolated after 12 h of incubation. The oligonucleotide GRR2 (indicated in Fig. 2a and 3), which was complementary to the proximal part of the *arcD* gene, served as a primer for precisely mapping the 5' end of *arc* mRNA and also for generating a matching sequence ladder. The single signal observed after primer extension (data not shown) placed the

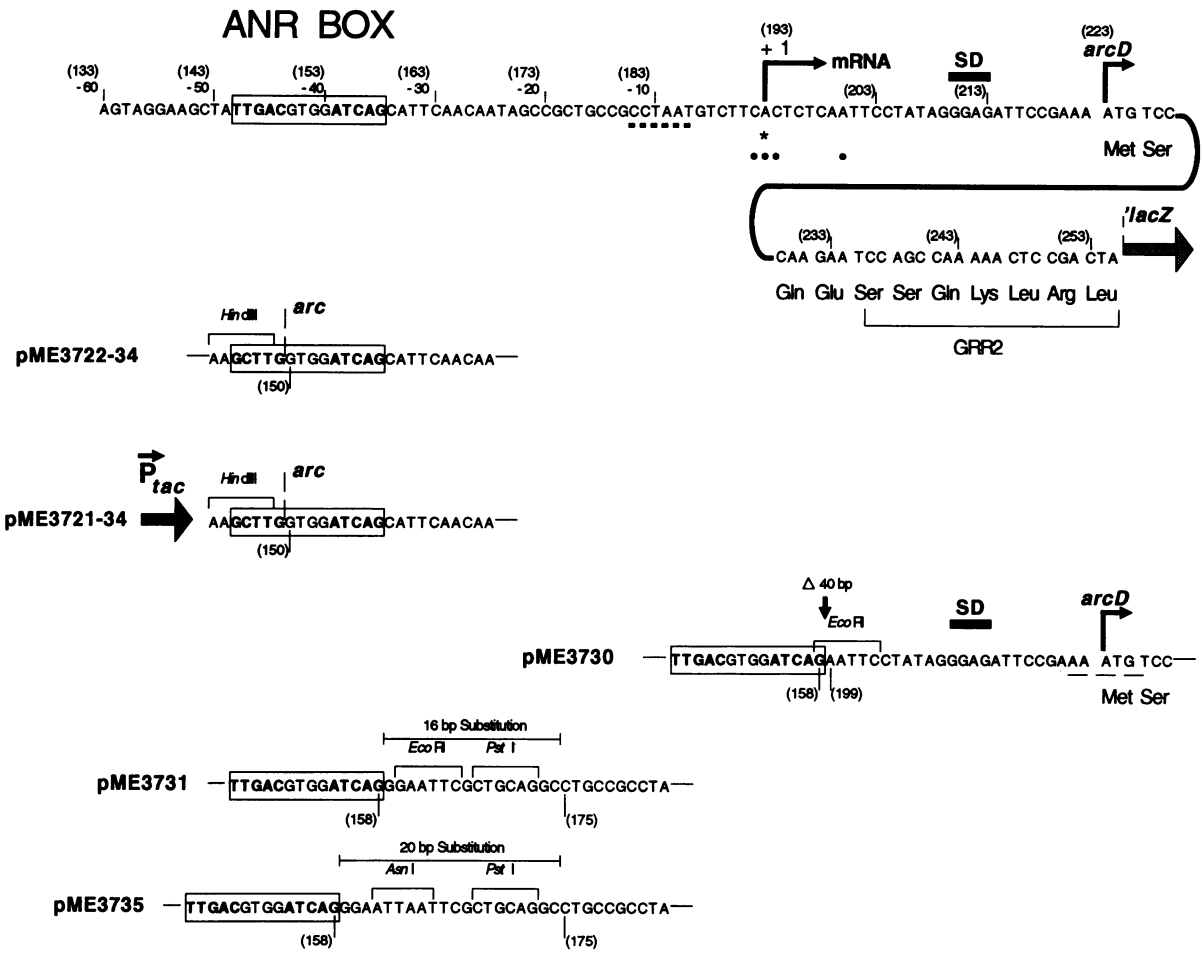


FIG. 3. Sequence of the wild-type *arc* promoter region, ANR box, and promoter mutations. All plasmids with promoter mutations carry all of the structural genes *arcDABC*; in pME3721-34, the *arc* operon (*Hin*dIII-*Sma*I) was cloned behind the *tac* promoter (P_{tac}) of pMMB67HE; the other plasmids are analogous to pME190, which has an insert of the wild-type *arc* operon (29). The 5′ ends of *arc* mRNAs obtained from anaerobic cultures by primer extension or S1 mapping (Fig. 2b) are indicated by * or ●, respectively. The +1 site was defined as the 5′ end of *arc* mRNA determined by primer extension. The ANR recognition sequence is boxed, and the putative −10 region is underlined with a dashed line. The numbers within parentheses indicate the distance in nucleotides from the *Hin*dIII site (Fig. 1) of the wild-type *arc* operon. The sequence complementary to the GRR2 primer (Fig. 2a) is marked. For the construction of the translational *arcD′-′lacZ* fusion of pME336 (Table 2), the *Mae*I site (Fig. 2a) at position 253 (C′TAG) was filled in with Klenow polymerase. For the plasmids with promoter mutations, only deviations from the wild-type sequence are shown. Δ, deletion; SD, Shine-Dalgarno region.

transcription start point at the A residue of position 193 (indicated by an asterisk in Fig. 3). In *E. coli* (21) and *P. aeruginosa* (11), A is the preferred residue for initiation of transcription (+1 site). Translation of the *arcD* gene is initiated 30 nucleotides downstream from the +1 site (28).

Inspection of the *arcD* promoter shows a −10 region (CCTAAT) that departs in the first two nucleotides from the consensus −10 sequence (TATAAT) recognized by σ^{70} RNA polymerase in *E. coli* (21) (Fig. 3). Deviations from the σ^{70} canonical sequence are not uncommon in positively controlled promoters of *E. coli* (38, 51, 55) and *P. aeruginosa* (11). No typical −35 sequence was found in the *arcD* promoter (Fig. 3), as in most other positively regulated promoters (11, 38). The center of the ANR recognition sequence (TTGAC....ATCAG) was located 41.5 bp upstream of the transcription start site (Fig. 3). In FNR-dependent promoters of *E. coli*, exactly the same spacing occurs between the FNR recognition sequence and the +1 site (4, 55). Mutational changes (deletion or insertion) in the −40 region of the *arc* operon are known to reduce strongly the anaerobic expression of ADI (18).

ANR-dependent control of transcription. Since ANR and FNR are homologous proteins (56) and since the ANR and FNR recognition sequences are very similar, it was assumed that ANR, like FNR, is a transcriptional activator. We have now tested this hypothesis directly by analyzing mRNAs in dot blot hybridization experiments. A mutant of *P. aeruginosa*, PAO6251, in which the entire *arc* operon is deleted (Materials and Methods), was chosen as a host strain for mRNA analysis. Plasmid pME190, which carries the *arc* operon on a 5.3-kb *Hin*dIII-*Sma*I fragment (Fig. 1) in the broad-host-range vector pKT240, provided the positive control. In strain PAO6251(pME190), *arcD* and *arcA* mRNAs were readily detected after oxygen had become growth limiting in the culture medium (Fig. 4), and ADI was induced to 195 U/mg of protein under these conditions. The deletion derivative pME3722-34, which is similar to pME190 but lacks the upstream half of the ANR box (Fig. 3), specified

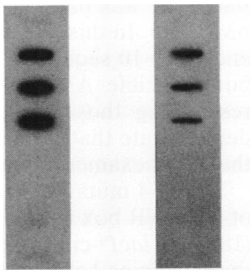
Plasmid in strain PAO6251	Incubation without aeration (h)	Cell growth ($OD_{600\ nm}$)	*arcD* mRNA	*arcA* mRNA
pME190	2.25	0.075		
	3.25	0.145		
	4.25	0.185		
	5.5	0.210		
pME3722-34	2.25	0.075		
	3.25	0.130		
	4.25	0.140		
	5.5	0.140		

FIG. 4. Effect of a deletion removing half of the ANR box on the anaerobic transcription of the *arc* operon. The deletion in pME3722-34 is shown in Fig. 3. For hybridization, 3.0×10^6 cpm of uniformly labeled anti-*arcD* RNA (from pME3701) or 3.5×10^5 cpm of uniformly labeled anti-*arcA* RNA (from pME3702) was utilized. At the time points indicated, samples were withdrawn from cultures of PAO6251(pME190) (wild-type *arc* operon) or PAO6251 (pME3722-34); the optical density at 600 nm was determined, and total RNA was isolated and subjected to dot blot analysis as described in Materials and Methods.

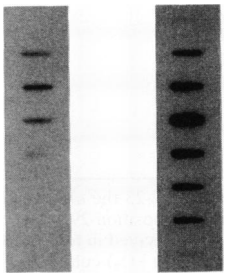
Incubation without aeration (h)	Cell growth ($OD_{600\ nm}$)	Sp. act. of ADI (U/mg)	*arcD* mRNA	*arcA* mRNA
2	ND	3		
2.25	0.095	ND		
2.5	0.105	ND		
2.75	ND	ND		
3	0.115	23		
4	0.130	33		
5	0.150	26		
23	0.375	26		

FIG. 5. Anaerobic induction of ADI and *arc* transcripts. ND, not determined. The specific activity of ADI was measured in 2.0-ml samples of toluenized cells. The experimental techniques for mRNA dot blotting were similar to those used in the experiments shown in Fig. 4., except that the same amount of radioactivity (2.6×10^6 cpm) was present in the *arcD* and *arcA* antisense RNAs.

low, almost noninducible ADI activity (19 U/mg of protein) in strain PAO6251. Dot blot analysis clearly showed that strain PAO6251(pME3722-34) produced very little *arcD* and *arcA* mRNAs after the onset of oxygen limitation (Fig. 4). The dot blot signals were quantified by scanning. The *arc* mRNA concentrations derived from pME3722-34 amounted to about 3% of those obtained from the wild-type *arc* plasmid pME190. Thus, the deletion mutation in the ANR box drastically lowered transcription of the *arc* operon, and the low levels of *arc* mRNA correlated with the low ADI activities. It can also be seen from Fig. 4 that lack of aeration led to a complete stop of growth (as determined by monitoring the optical density at 600 nm) in strain PAO6251(pME3722-34), whereas the *arc*$^+$ plasmid pME190 in strain PAO6251 allowed slow anaerobic growth with arginine as the energy source, as previously shown (52).

Additional evidence for ANR-dependent transcription of the *arc* operon came from RNA analysis of the *anr* mutant S1239. In this strain, ADI is not inducible by limited aeration (18). No *arc*-specific mRNA could be detected when total RNA was extracted from an oxygen-limited culture of strain S1239 and analyzed by the dot blot method (data not shown). In contrast, *arc*-specific mRNA could be detected readily in an analogous experiment with RNA extracted from the wild-type strain PAO1 (Fig. 5).

Taken together, these results show that transcription of the *arc* operon during oxygen limitation depends on ANR and an intact ANR recognition sequence.

Time course of induction. We wished to follow the formation of *arcD* and *arcA* transcripts in the wild-type PAO1 during the transition from aerobic to anaerobic growth conditions. In parallel we monitored the expression of ADI and that of two translational gene fusions, *arcD'-'lacZ* and *arcA'-'lacZ*. In the construction of the *arcD'-'lacZ* fusion plasmid pME336 (Fig. 3), care was taken to fuse β-galactosidase closely to the N terminus of the ArcD protein. ArcD is a highly hydrophobic protein with 13 segments that could span the cytoplasmic membrane; the first potential transmembrane segment of ArcD starts with Leu-11 (28). The *arcD'-'lacZ* construct pME336 carried the 11 N-terminal codons of *arcD* fused, via a linker, to the ninth codon of *lacZ*. Thus, we expect the ArcD-LacZ hybrid enzyme to be a soluble protein with good β-galactosidase activity. The *arcA'-'lacZ* fusion plasmid pME340 has been described previously (56).

An aerobically growing culture of strain PAO1 was shifted to oxygen limitation by shutting off the air supply at time zero. We know from earlier experiments that under such conditions oxygen is rapidly consumed by respiration and that the enzymes of the ADI pathway are induced when the oxygen tension is strongly reduced (32). Here we observed induction of ADI after 2 to 4 h of incubation without aeration (Table 2; Fig. 5). In the same time interval, the β-galactosidase activities specified by both the *arcD'-'lacZ* and the *arcA'-'lacZ* fusion plasmids pME336 and pME340 in strain PAO1 increased steeply (Table 2). From this experiment we conclude that the *arcD* and *arcA* genes are coordinately regulated, as would be expected for an operonic arrangement of these genes (28). The specific activities of the ArcD-LacZ β-galactosidase were higher than those of the

TABLE 2. Expression of *arcD'-'lacZ* and *arcA'-'lacZ* translational fusions

Plasmid in strain PAO1	Incubation[a] time (h)	Cell growth (OD_{600}[b])	β-Galactosidase activity[c] (kU)	Sp act of ADI (U/mg)
pME336 (*arcD'-'lacZ*)	2	0.065	3.4	4
	3	0.150	7.6	12
	4	0.165	15.8	51
	5	0.185	25.7	43
	6	0.190	34.7	37
pME340 (*arcA'-'lacZ*)	2	0.045	1.8	2
	3	0.095	2.9	2
	4	0.130	6.1	22
	5	0.130	7.8	28
	6	0.140	7.3	36

[a] Cells were incubated without aeration.
[b] OD_{600}, optical density at 600 nm.
[c] β-Galactosidase was measured in toluenized cells.

TABLE 3. Effects of mutations in the *arc* promoter region on the anaerobic induction of ADI in PTO6235 (*arcA*::*mer*)

Plasmid[a]	Sp act of ADI[b]	
	$+O_2$	$-O_2$
pME190	19[c]	216[c]
pME3722-23	1	6
pME3730	4	32
pME3731	35	222
pME3735	3	34
pME3722-34	4	14
pME3721-34	180[d]	209[d]

[a] In pME3722-23 the *arc* promoter region is deleted from the *Hin*dIII site (position 1) to position 292. The other constructs are shown in Fig. 3.
[b] ADI was assayed in toluenized cells obtained from well-aerated ($+O_2$) or oxygen-limited ($-O_2$) cultures.
[c] Values taken from Galimand et al. (18).
[d] Values obtained after isopropyl-β-D-thiogalactoside induction.

ArcA-LacZ enzyme (Table 2). The reasons for this difference are not clear.

In a parallel shift experiment, *arcD* and *arcA* transcripts were quantitated by dot blotting (Fig. 5) and scanning measurements. After 2.0 to 2.75 h of incubation without aeration, *arc* mRNA levels rose sharply. The concentration of *arcD* mRNA increased by a factor of about 10 between 2.0 and 2.5 h, and that of *arcA* mRNA increased by a factor of 20 to 30 between 2.0 and 2.75 h (Fig. 5). These increases in *arc* transcripts appeared to be specific in that the transcripts of the constitutive *aph* (kanamycin resistance) gene of pKT240 were produced at uniform levels in a control experiment (not shown). After 3 to 5 h of incubation, the levels of the *arc* transcripts decreased (Fig. 5), indicating that de novo synthesis of the *arc* transcripts was gradually reduced to an uninduced state. The fact that *arcA* mRNA was detectable for a longer time than was *arcD* mRNA might be a consequence of differential mRNA stabilities. However, this point was not investigated further.

Effects of mutations in the *arc* promoter region. The consequences of mutational changes in the *arc* promoter region were assessed by measuring specific ADI activities that were expressed by pME190 derivatives consisting of a mutated *arc* promoter, the entire *arc* operon, and the vector pKT240. The host strain was PTO6235 (*arcA*::*mer*). The wild-type promoter on pME190 was included as a control (Table 3). All plasmids tested had similar copy numbers, as judged from similar yields of plasmid DNA in cleared lysates. When the entire *arc* promoter region and the proximal part of the *arcD* gene (to position 292 relative to the unique *Hin*dIII site [Fig. 1]) were deleted in pME3722-23, very low ADI activities were detected (Table 3), suggesting that it is unlikely that an additional downstream promoter exists (e.g., in the distal part of *arcD*). Oligonucleotide mutagenesis was used to construct promoter mutations in pME3730 and pME3731 (Fig. 3). In pME3730, 40 bp between the ANR box and the presumed ribosome binding site of the *arcD* gene were deleted. This deletion created, around the translation initiation site of *arcD*, a rather poor −10 sequence (AAATGT; Fig. 3). As a result, low ADI activities were measured. However, some anaerobic induction could still be observed (Table 3), presumably because the ANR box was left intact.

In pME3731, the 16 bp immediately downstream of the ANR box were substituted by a 16-bp *Eco*RI-*Pst*I linker sequence. The linker was different from the original sequence at every base, but spacing between the ANR box and the −10 region was conserved (Fig. 3). The substitution had no effect on promoter strength or anaerobic induction (Table 3). The −35 region, therefore, does not appear to be critical for *arc* promoter function. The newly created *Eco*RI site of pME3731 was filled in with Klenow polymerase, producing pME3735. In this plasmid the spacing between the ANR box and the −10 sequence was increased by 4 bp (Fig. 3). Low but inducible ADI levels could be determined (Table 3), resembling those obtained with pME3730. These results demonstrate that proper spacing between the ANR box and the −10 hexamer is important for promoter strength.

The Δ34 mutation, which removes the upstream half-site of the ANR box in pME3722-34, has already been described (18). The *lacI*^{q}-controlled *tac* promoter was fused directly to the remaining half-site of the ANR box in pME3721-34 (Fig. 3). *P. aeruginosa* cultures carrying this plasmid were induced with isopropyl-β-D-thiogalactoside. During conditions of limited aeration, the induced *tac* promoter of pME3721-34 and the wild-type *arc* promoter of pME190 both specified similar ADI levels (Table 3). Since it is clear that the *tac* promoter functions very well in *P. aeruginosa* (44), we conclude that the *arc* promoter activated by ANR can also be ranked among the effective *Pseudomonas* promoters.

Anaerobic regulation of the *arc* operon did not operate in strain PTO6235(pME3721-34), as shown by the high ADI activity in a well-aerated culture (Table 3). This control further implies that the catalytic activity of ADI is not affected by oxygen, i.e., that ADI assays are indeed suitable for measuring transcriptional regulation of the *arc* operon.

DISCUSSION

In previous studies (18, 56), we compared ANR and FNR in terms of primary structure and regulatory function. These proteins have very similar molecular weights and display considerable amino acid sequence identity, particularly in their helix-turn-helix motifs, which are important for the recognition of the ANR and FNR boxes, respectively (56). ANR and FNR have key roles in the expression of anaerobic metabolism; both proteins are positive regulators of nitrate respiration in their native hosts. Control of fumarate reduction is another important function of FNR in *E. coli* (46). In contrast, *P. aeruginosa* cannot grow anaerobically on fumarate but is able to use arginine as an energy source in the absence of respiration (52). ANR controls anaerobic arginine utilization by positively regulating the expression of the *arc* operon (18). The consensus FNR box (TTGAT...ATCAA) allows good anaerobic, ANR-dependent expression in *P. aeruginosa*, whereas the ANR box (TTGAC....ATCAG) of the *arc* operon appears to be only partially recognized by FNR in *E. coli* (18). Thus, the recognition specificities of the two regulators may be quite similar but not identical.

In the present work, we demonstrate that ANR acts as a transcriptional activator. First, a deletion in the ANR box of the *arc* operon strongly reduced transcription of the operon, and a mutation in the *anr* gene had the same effect. Second, when oxygen became limiting, the wild-type strain PAO1 showed a parallel increase in *arcDA* mRNA concentrations and in the expression of the ArcD and ArcA proteins.

The *arc* operon was expressed even under aerobic conditions. Part of this expression appears to be due to ANR-activated transcription because an *anr* mutation reduces about threefold the aerobic activities of ADI encoded by the wild-type *arc* operon and those of β-galactosidase expressed from an FNR consensus promoter (18). Moreover, the deletion in the ANR box of pME3722-34 also affected

aerobic ADI expression (Table 3). The residual, basal activity of ADI might be due to a second promoter overlapping with the ANR-activated promoter. Preliminary evidence for this comes from additional 5′ ends of *arc* mRNA when RNA was extracted from aerobic, stationary-phase cultures (data not shown).

The current model of FNR-dependent transcription postulates that under reduced oxygen tension FNR binds to the FNR box and thereby activates σ^{70} RNA polymerase, which demands a reasonable (although not perfect) −10 hexamer at an appropriate distance from the FNR box; the nucleotide sequence in the −35 region is not critical (4, 46, 55). This model has recently been confirmed by in vitro experiments (19a); moreover, it is amply supported by in vivo experiments and by parallel studies on the analogous regulator CRP (cyclic AMP receptor protein) (19, 45, 51). The transcriptional data of this study suggest that the same model can be used to describe ANR-dependent transcription; the −10 region, but not the −35 region, of the *arc* promoter was found to be important for ANR-dependent expression of the *arc* operon.

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REFERENCES

1. **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.
2. **Baur, H., E. Lüthi, V. Stalon, A. Mercenier, and D. Haas.** 1989. Sequence analysis and expression of the arginine-deiminase and carbamate-kinase genes of *Pseudomonas aeruginosa*. Eur. J. Biochem. **179**:53–60.
3. **Baur, H., V. Stalon, P. Falmagne, E. Lüthi, and D. Haas.** 1987. Primary and quaternary structure of the catabolic ornithine carbamoyltransferase from *Pseudomonas aeruginosa*. Eur. J. Biochem. **166**:111–117.
4. **Bell, A. I., J. A. Cole, and S. J. W. Busby.** 1990. Molecular genetic analysis of an FNR-dependent anaerobically inducible *Escherichia coli* promoter. Mol. Microbiol. **4**:1753–1763.
5. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. Betlach, H. L. Heyneker, H. W. Boyer, J. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2**:95–113.
6. **Bourdineaud, J.-P., H. Verhoogt, and D. Haas.** Unpublished data.
7. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from P15A cryptic miniplasmid. J. Bacteriol. **134**:1141–1156.
8. **Comai, L., C. Schilling-Cordaro, A. Mergia, and C. M. Houck.** 1983. A new technique for genetic engineering of *Agrobacterium* Ti plasmid. Plasmid **10**:21–30.
9. **Davies, K. J. P., D. Lloyd, and L. Boddy.** 1989. The effect of oxygen on denitrification in *Paracoccus denitrificans* and *Pseudomonas aeruginosa*. J. Gen. Microbiol. **135**:2445–2451.
10. **Del Sal, G., G. Manfioletti, and C. Schneider.** 1988. A one-tube plasmid DNA mini-preparation suitable for sequencing. Nucleic Acids Res. **16**:9878.
11. **Deretic, V., W. M. Konyecsni, C. D. Mohr, D. W. Martin, and N. S. Hibler.** 1989. Common denominators of promoter control in *Pseudomonas* and other bacteria. Bio/Technology **7**:1249–1254.
12. **Eiglmeier, K., N. Honoré, S. Iuchi, E. C. C. Lin, and S. T. Cole.** 1989. Molecular genetic analysis of FNR-dependent promoters. Mol. Microbiol. **3**:869–878.
13. **Favaloro, J., R. Treisman, and R. Kamen.** 1980. Transcription maps of polyoma virus-specific RNA: analysis by two-dimensional nuclease S1 gel mapping. Methods Enzymol. **65**:718–749.
14. **Fellay, R., J. Frey, and H. Krisch.** 1987. Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for *in vitro* insertional mutagenesis of Gram-negative bacteria. Gene **52**:147–154.
15. **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.
16. **Früh, R., J. M. Watson, and D. Haas.** 1983. Construction of recombination-deficient strains of *Pseudomonas aeruginosa*. Mol. Gen. Genet. **191**:334–337.
17. **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 *tacP* expression vector. Gene **48**:119–131.
18. **Galimand, M., M. Gamper, A. Zimmermann, and D. Haas.** 1991. Positive FNR-like control of anaerobic arginine degradation and nitrate respiration in *Pseudomonas aeruginosa*. J. Bacteriol. **173**:1598–1606.
19. **Gaston, K., A. Bell, A. Kolb, H. Buc, and S. Busby.** 1990. Stringent spacing requirements for transcription activation by CRP. Cell **62**:733–743.

19a. **Green, J., M. Trageser, S. Six, G. Unden, and J. R. Guest.** 1991. Characterization of the FNR protein of *Escherichia coli*, an iron-binding transcriptional regulator. Proc. R. Soc. London B **244**:137–144.

20. **Haas, D., J. Watson, R. Krieg, and T. Leisinger.** 1981. Isolation of an Hfr donor of *Pseudomonas aeruginosa* PAO by insertion of the plasmid RP1 into the tryptophan synthase gene. Mol. Gen. Genet. **182**:240–244.
21. **Harley, C. B., and R. P. Reynolds.** 1987. Analysis of *E. coli* promoter sequences. Nucleic Acids Res. **15**:2343–2361.
22. **Hennecke, H., I. Günther, and F. Binder.** 1982. A novel cloning vector for the direct selection of recombinant DNA in *E. coli*. Gene **19**:231–234.
23. **Holloway, B. W., K. O'Hoy, and H. Matsumoto.** 1987. *Pseudomonas aeruginosa* PAO, p. 213–221. *In* S. J. O'Brien (ed.) Genetic maps, vol. 4. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Hopwood, D. A., M. J. Bibb, K. F. Chater, T. Kieser, C. J. Bruton, H. M. Kieser, D. J. Lydiate, C. P. Smith, J. M. Ward, and H. Schrempf.** 1985. Genetic manipulation of *Streptomyces*. A laboratory manual. The John Innes Foundation, Norwich, United Kingdom.
25. **Itoh, Y., J. M. Watson, D. Haas, and T. Leisinger.** 1984. Genetic and molecular characterization of the *Pseudomonas* plasmid pVS1. Plasmid **11**:206–220.
26. **Kiley, P. A., and W. S. Reznikoff.** 1991. Fnr mutants that activate gene expression in the presence of oxygen. J. Bacteriol. **173**:16–22.
27. **Kullik, I., H. Hennecke, and H.-M. Fischer.** 1989. Inhibition of *Bradyrhizobium japonicum nifA*-dependent *nif* gene activation by oxygen occurs at the NifA protein level and is irreversible. Arch. Microbiol. **151**:191–197.
28. **Lüthi, E., H. Baur, M. Gamper, F. Brunner, D. Villeval, A. Mercenier, and D. Haas.** 1990. The *arc* operon for anaerobic arginine catabolism in *Pseudomonas aeruginosa* contains an additional gene, *arcD*, encoding a membrane protein. Gene **87**:37–43.
29. **Lüthi, E., A. Mercenier, and D. Haas.** 1986. The *arcABC* operon required for fermentative growth of *Pseudomonas aeruginosa* on arginine: Tn*5-751*-assisted cloning and localization of structural genes. J. Gen. Microbiol. **132**:2667–2675.
30. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12**:7035–7056.

31. **Melville, S. B., and R. P. Gunsalus.** 1990. Mutations in *fnr* that alter anaerobic regulation of electron transport-associated genes in *Escherichia coli*. J. Biol. Chem. **265**:18733–18736.
32. **Mercenier, A., J.-P. Simon, C. Vander Wauven, D. Haas, and V. Stalon.** 1980. Regulation of enzyme synthesis in the arginine deiminase pathway of *Pseudomonas aeruginosa*. J. Bacteriol. **144**:159–163.
33. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101**:20–78.
34. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
35. **Minton, N. P.** 1984. Improved plasmid vectors for the isolation of translational *lac* gene fusions. Gene **31**:269–273.
36. **Morgan, A. F.** 1979. Transduction of *Pseudomonas aeruginosa* with a mutant of bacteriophage E79. J. Bacteriol. **139**:137–140.
37. **Palleroni, N. J.** 1984. Family I. *Pseudomonadaceae* Winslow, Broadhurst, Buchanan, Krumwiede, Rogers, and Smith 1917, 555, p. 141–219. *In* N. R. Krieg (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins, Co., Baltimore.
38. **Raibaud, O., and M. Schwartz.** 1984. Positive control of transcription initiation in bacteria. Annu. Rev. Genet. **18**:173–206.
39. **Reimmann, C., M. Rella, and D. Haas.** 1988. Integration of replication-defective R68.45-like plasmids into the *Pseudomonas aeruginosa* chromosome. J. Gen. Microbiol. **134**:1515–1523.
40. **Rella, M., A. Mercenier, and D. Haas.** 1985. Transposon insertion mutagenesis of *Pseudomonas aeruginosa* with a Tn*5* derivative: application to physical mapping of the *arc* gene cluster. Gene **33**:293–303.
41. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
42. **Short, J. M., J. M. Fernandez, J. A. Sorge, and W. D. Huse.** 1988. λ ZAP: a bacteriophage λ expression vector with *in vivo* excision properties. Nucleic Acids Res. **16**:7583–7600.
43. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1**:784–791.
44. **Soldati, L., D. J. Jeenes, and D. Haas.** 1987. Effective gene expression in *Pseudomonas aeruginosa* under the control of the *Escherichia coli* consensus promoter. FEMS Microbiol. Lett. **42**:163–167.
45. **Spiro, S., K. L. Gaston, A. I. Bell, R. E. Roberts, S. J. W. Busby, and J. R. Guest.** 1990. Interconversion of the DNA-binding specificities of two related transcription regulators, CRP and FNR. Mol. Microbiol. **4**:1831–1838.
46. **Spiro, S., and J. R. Guest.** 1990. FNR and its role in oxygen-regulated gene expression in *Escherichia coli*. FEMS Microbiol. Rev. **75**:399–428.
47. **Spiro, S., R. E. Roberts, and J. R. Guest.** 1989. FNR-dependent repression of the *ndh* gene of *Escherichia coli* and metal ion requirement for FNR-regulated gene expression. Mol. Microbiol. **3**:601–608.
48. **Stanisich, V. A., and B. W. Holloway.** 1972. A mutant sex factor of *Pseudomonas aeruginosa*. Genet. Res. **19**:91–108.
49. **Taylor, J. W., J. Ott, and F. Eckstein.** 1985. The rapid generation of oligonucleotide-directed mutations at high frequency using phosphorothioate-modified DNA. Nucleic Acids Res. **13**:8765–8785.
50. **Ubben, D., and R. Schmitt.** 1986. Tn*1721* derivatives for transposon mutagenesis, restriction mapping and nucleotide sequence analysis. Gene **41**:145–152.
51. **Valentin-Hansen, P., B. Holst, L. Sogaard-Andersen, J. Martinussen, J. Nesvera, and S. R. Douthwaite.** 1991. Design of cAMP-CRP-activated promoters in *Escherichia coli*. Mol. Microbiol. **5**:433–437.
52. **Vander Wauven, C., A. Piérard, M. Kley-Raymann, and D. Haas.** 1984. *Pseudomonas aeruginosa* mutants affected in anaerobic growth on arginine: evidence for a four-gene cluster encoding the arginine deiminase pathway. J. Bacteriol. **160**:928–934.
53. **Van Hartingsveldt, J., M. G. Marinus, and A. H. Stouthamer.** 1971. Mutants of *Pseudomonas aeruginosa* blocked in nitrate or nitrite dissimilation. Genetics **67**:469–482.
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. **Walker, M. S., and J. A. DeMoss.** 1991. Promoter sequence requirements for Fnr-dependent activation of transcription of the *narGHJI* operon. Mol. Microbiol. **5**:353–360.
56. **Zimmermann, A., C. Reimmann, M. Galimand, and D. Haas.** Mol. Microbiol., in press.