APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1996, p. 3391–3398 Vol. 62, No. 9
0099-2240/96/$04.00+0

Anaerobically Controlled Expression System Derived from the *arcDABC* Operon of *Pseudomonas aeruginosa*: Application to Lipase Production

HARALD V. WINTELER,[1] BERND SCHNEIDINGER,[2] KARL-ERICH JAEGER,[2] AND DIETER HAAS[1]*

Laboratoire de Biologie Microbienne, Université de Lausanne, CH-1015 Lausanne, Switzerland,[1] and Lehrstuhl für Biologie der Mikroorganismen, Ruhr-Universität, D-44780 Bochum, Germany[2]

Received 12 March 1996/Accepted 30 June 1996

The anaerobically inducible *arcDABC* operon encodes the enzymes of the arginine deiminase pathway in *Pseudomonas aeruginosa*. Upon induction, the *arcAB* mRNAs and proteins reach high intracellular levels, because of a strong anaerobically controlled promoter and mRNA processing in *arcD*, leading to stable downstream transcripts. We explored the usefulness of this system for the construction of expression vectors. The *lacZ* gene of *Escherichia coli* was expressed to the highest levels when fused close to the *arc* promoter. Insertion of *lacZ* further downstream into *arcA* or *arcB* did not stabilize the intrinsically unstable *lacZ* mRNA. On the contrary, *lacZ* mRNA appeared to be a vulnerable endonuclease target destabilizing *arcAB* mRNAs in the 5′-to-3′ direction in *P. aeruginosa*. The native *arc* promoter was modified for optimal expression in the −10 sequence and in the −40 region, which is a binding site for the anaerobic regulator ANR. In *P. aeruginosa* grown either anaerobically or with oxygen limitation in unshaken cultures, this promoter was stronger than the induced *tac* promoter. The *P. aeruginosa lipAH* genes, which encode extracellular lipase and lipase foldase, respectively, were fused directly to the modified *arc* promoter in an IncQ vector plasmid. Semianaerobic static cultures of *P. aeruginosa* PAO1 carrying this recombinant plasmid overproduced extracellular lipase 30-fold during stationary phase compared with the production by strain PAO1 without the plasmid. Severe oxygen limitation, in contrast, resulted in poor lipase productivity despite effective induction of the ANR-dependent promoter, suggesting that secretion of active lipase is blocked by the absence of oxygen. In conclusion, the modified *arc* promoter is useful for driving the expression of cloned genes in *P. aeruginosa* during oxygen-limited growth and stationary phase.

Pseudomonas aeruginosa is an aerobic bacterium which adapts readily to anoxic conditions, using either nitrate respiration or arginine fermentation to generate metabolic energy (19, 50, 58). Anaerobic degradation of L-arginine depends on the arginine deiminase (ADI) pathway encoded by the *arcDABC* operon and the FNR-like anaerobic regulator ANR (13, 32, 50, 59). Oxygen limitation strongly and coordinately induces the cytoplasmic enzymes of the ADI pathway: ADI, catabolic ornithine carbamoyltransferase (cOTC), and carbamate kinase (Fig. 1) (33, 36). When the *arc* operon is carried by a multicopy IncQ plasmid, ADI (the *arcA* product) and cOTC (the *arcB* product) are the most abundant proteins under inducing conditions and represent >20% of the total cellular protein, whereas carbamate kinase (the *arcC* product) is synthesized in smaller amounts (3, 4). The ArcD protein, a transmembrane arginine-ornithine exchanger and the product of the *arcD* gene, is produced sparingly (6, 32, 51).

High-level expression of the *arcA* and *arcB* genes depends essentially on two mechanisms. First, the transcriptional activator ANR, activated by oxygen limitation (36, 59), effectively stimulates transcription of the *arc* operon by binding to the Arc box TTGAC....ATCAG, which is located at position −40 from the transcription start site (15, 17, 54) (Fig. 1). Disruption of the *anr* gene or mutational changes in the Arc box abolish transcriptional activation of the *arc* operon (13, 54, 59). Second, RNA processing results in the formation of relatively stable *arcABC*, *arcAB*, and *arcA* transcripts having chemical half-lives of 13 to 16 min (15). An RNase E-like enzyme cuts in the distal part of *arcD* (see Fig. 2B), leading to enhanced degradation of the *arcD* segment at the 5′ end of the primary *arc* transcripts (15). The 3′ ends of the *arc* transcripts are determined by stable stem-loop structures located downstream of *arcA*, *arcB*, and *arcC*. The hairpin structure at the 3′ end of *arcC* forms a rho-independent terminator (see Fig. 2B). The expression levels of the *arcDABC* gene products correlate approximately with the relative abundance of the corresponding *arc* transcripts, in the order *arcA* (highest)-*arcB*-*arcC*-*arcD* (lowest) (15).

Deletion of either the processing sites in *arcD* or the stem-loop structures downstream of *arcA* or *arcB* lowers the levels of expression of ADI and cOTC, apparently by decreasing the amounts of the corresponding transcripts (15). This observation implies that the *arcA* and *arcB* transcripts owe their stability, at least in part, to sequences located at their 5′ ends as well as at their 3′ ends. In general, bacterial mRNAs are degraded rapidly but can be stabilized by 5′ stabilizers and/or stem-loop structures located at the 3′ ends of transcripts (10, 23, 42, 43).

The anaerobically inducible, highly efficient expression of the *P. aeruginosa arcA* gene has prompted us to exploit this system for the construction of expression vectors in *Pseudomonas* spp. Anaerobically activated promoters (28, 38, 40) and mRNA stabilizers placed at either the 5′ or the 3′ end (22, 56) have been used previously to enhance the expression of heterologous genes in enteric bacteria. In the first part of this study, we investigated the utility of ANR-dependent promoters

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

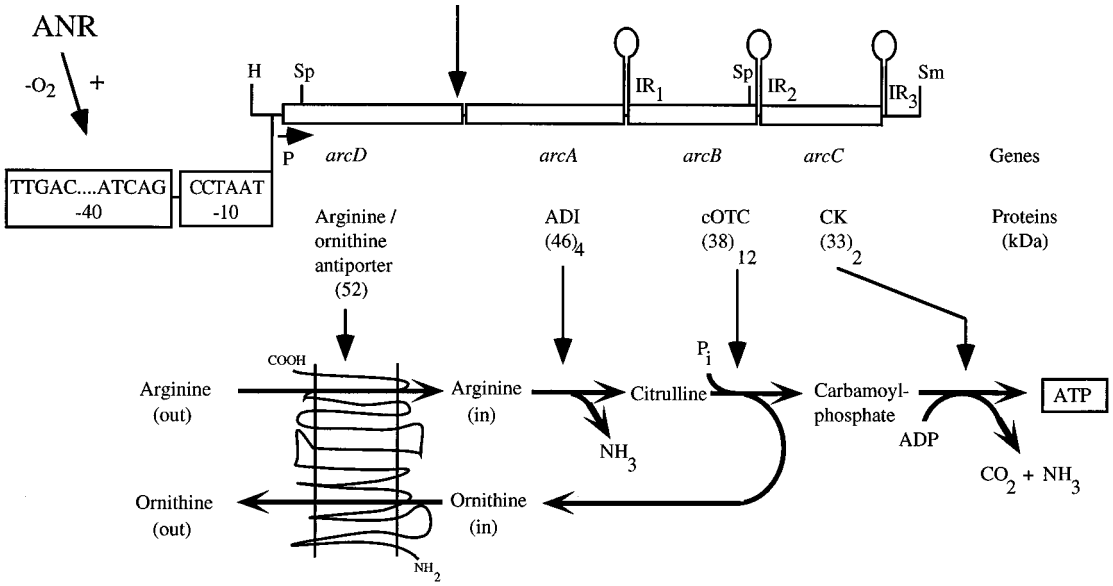

FIG. 1. The *arcDABC* operon (5.0 kb), its gene products, and the ADI pathway of *P. aeruginosa*. The anaerobically inducible *arc* promoter (P) is shown with the −40 region (the ANR binding site) and the −10 region. Inverted repeats IR_1 and IR_2 specify stem-loop structures in the *arc* transcripts. They constitute barriers against 3′-exonuclease attack and/or weak transcription terminators. IR_3 is a rho-independent terminator (15). The site(s) of mRNA processing (15, 16) (vertical arrow) is indicated. CK, carbamate kinase. Restriction sites: H, *Hin*dIII; Sp, *Sph*I; and Sm, *Sma*I.

and stabilizing segments from *arc* transcripts to construct vectors for regulated, high-level expression of cloned genes in *P. aeruginosa* and other species of fluorescent pseudomonads.

In the second part, we show that a newly developed, ANR-dependent expression system allows overexpression of lipase from *P. aeruginosa*. Bacterial lipases, especially those from the genus *Pseudomonas*, are of biotechnological interest because of their usefulness as additives in household detergents or as catalysts for the resolution of racemic compounds which serve as building blocks in the synthesis of pharmaceuticals and agrochemicals (25). *Pseudomonas* lipase cannot be secreted by *Escherichia coli*, and when overproduced in this host, the enzyme accumulates as inclusion bodies (39). Therefore, it is preferable to use the native *Pseudomonas* host for lipase production. Ideally, the complex secretion machinery encoded by *xcp* genes should be coexpressed with lipase. The *xcp* genes of *P. aeruginosa* are induced naturally during the transition from exponential to stationary phase (1). In parallel, the ANR-dependent expression system reported here can be turned on by oxygen limitation, i.e., by conditions prevailing in unshaken, stationary-phase cultures. Thus, by expressing the lipase structural genes *lipAH* under ANR control, we have achieved a simple and inexpensive procedure for enhanced production of extracellular lipase.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used are listed in Table 1.

Media and growth conditions. All incubations were carried out at 37°C. The liquid and solid media for aerobic growth of *E. coli* and *P. aeruginosa* were nutrient yeast broth, nutrient agar, and minimal medium E (44). For oxygen-limited and anaerobic growth of *P. aeruginosa*, yeast extract-arginine (YEA) medium was used (50). Maintenance of pKT240 derivatives in *P. aeruginosa* was ensured by the addition of 250 μg of carbenicillin per ml. For *E. coli*, 100 μg of ampicillin per ml was used instead.

DNA manipulations. Standard techniques were used for restriction, agarose gel electrophoresis, dephosphorylation with alkaline phosphatase, nick translation with DNA polymerase I, Southern blotting, generation of blunt ends with Klenow polymerase, isolation of DNA fragments from low-melting-point agarose gels, ligation, and transformation by $CaCl_2$ treatment (45). *E. coli* XL1Blue was used for cloning experiments. Electroporation of *P. aeruginosa* with plasmid DNA was done as described previously (59). Small-scale plasmid preparations were performed by the method of Del Sal et al. (11). Qiagen purification (Diagen) was used to obtain large-scale plasmid DNA preparations. Chromosomal DNA of *P. aeruginosa* was isolated as described by Gamper et al. (15). Double-stranded DNA was sequenced by the chain termination method with Sequenase (U.S. Biochemical Corp.) according to the manufacturer's instructions.

Construction of chromosomal *arc-lacZ* mutants of *P. aeruginosa*. Transcriptional *lacZ* fusions at four different positions in the *arc* operon were stably integrated into the chromosome of *P. aeruginosa* PAO1 via the mobilizable, ColE1-derived suicide vector pME3087 (52, 58). The *Eco*RI site of pME3087 was removed by filling in with DNA polymerase I (Klenow fragment). The resulting plasmid, pME3087ΔE, was used to clone the 5.5-kb *Hin*dIII-*Sac*I fragment carrying the *arc* operon with an artificial *Eco*RI site in *arcA* (from pME190::ω1), *arcB* (from pME190::ω2), or *arcD* (from pME190::ω3) or the 5.3-kb *Hin*dIII-*Sac*I fragment carrying the *arc* operon with an artificial *Eco*RI site at the 3′ end of *arcD* (from pME3732) (Table 1). The promoterless *lacZ* gene derived from pML8 (31) was excised as a 3.2-kb *Eco*RI fragment from pME3517 (see Fig. 2A and B) and inserted into the *Eco*RI sites of the above constructs. The *lacZ* fusion plasmids were mobilized from *E. coli* S17-1 to *P. aeruginosa* PAO1 grown at 43°C (58). Selection for tetracycline resistance (on minimal medium E containing 50 μg of tetracycline per ml) at 37°C produced PAO1 transconjugants carrying a chromosomally integrated copy of the pME3087 derivatives at about 4×10^{-7} per donor. After enrichment with D-cycloserine to eliminate the integrated plasmid (20), tetracycline-sensitive, Lac^+ colonies (blue on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-Gal] [200 μg/ml]) were obtained. Chromosomal DNAs of these *arc-lacZ* mutants were digested with *Bam*HI and *Hin*dIII and hybridized against an *arc'DAB'* probe (3.6-kb *Sph*I fragment; Fig. 1). Southern blotting confirmed the *lacZ* insertion in *arcA* (PAO6263), *arcB* (PAO6264), and *arcD* (PAO6265 and PAO6266) (data not shown).

RNA experiments. Total RNA of the wild-type *P. aeruginosa* PAO1 and the four *arc-lacZ* mutants was isolated after 5 h of oxygen-limited growth in YEA medium as described elsewhere (15, 30). For Northern (RNA) blot analysis, digoxigenin (DIG)-labeled *arcA* antisense RNA was synthesized in vitro with the pGEM-1-derived plasmid pME3702 (Fig. 2B) as a template and T7 RNA polymerase and DIG-UTP in a reaction mix as specified by Boehringer (Mannheim, Germany). Electrophoretic separation on an agarose gel and transfer to a Hybond-N membrane (Amersham) were done as described previously (15). Prehybridization, hybridization against the DIG-labeled antisense RNA probe, and immunological detection were performed as specified by Boehringer.

Immunoblot analysis of lipase. The proteins in dialyzed culture supernatants were concentrated by evaporation under reduced pressure, dissolved in sample buffer (50 mM Tris-HCl [pH 6.8], 10% [vol/vol] glycerol, 2% [vol/vol] 2-mercaptoethanol, 0.03% bromophenol blue) containing 4% (wt/vol) sodium dodecyl sulfate (SDS), and loaded onto an SDS–12% (wt/vol) polyacrylamide gel for electrophoresis. Membrane and soluble fractions were obtained from *P. aeruginosa* cells harvested at various times of growth. Cells were resuspended in 5 ml

TABLE 1. Bacterial strains and plasmids

Strain	Genotype or relevant characteristics	Reference(s) or source
Strains		
P. aeruginosa		
PAO1	Wild type	24
PAO6251	Δ(*arcDABC*)	17
PAO6263	*arcA-lacZ* transcriptional fusion	This work (Fig. 2B)
PAO6264	*arcB-lacZ* transcriptional fusion	This work (Fig. 2B)
PAO6265	*arcD-lacZ* transcriptional fusion	This work (Fig. 2B)
PAO6266	*arcD-lacZ* transcriptional fusion	This work (Fig. 2B)
E. coli		
S17-1	*pro thi hsdR recA*; chromosomal insertion of RP4-2	46
XL1Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*/F′ *proAB lacI*^{q} *lacZ*ΔM15 Tn*10*	Stratagene
Plasmids		
pBluescript KS(+)	Ap/Cb ColE1 replicon (3.0 kb)	Stratagene
pKT240	Ap/Cb Km Mob IncQ (12.5 kb)	2
pME190	Ap/Cb Mob IncQ (16.1 kb); 5.3-kb *Hin*dIII-*Sma*I fragment carrying *arcDABC*, cloned into pKT240	33
pME190::ω-1	pME190 derivative with 35-bp insertion from Tn*1725* in *arcA*	14, 32
pME190::ω-2	pME190 derivative with 35-bp insertion from Tn*1725* in *arcB*	14
pME190::ω-3	pME190 derivative with 35-bp insertion from Tn*1725* in *arcD*	32
pME336	Ap/Cb Mob IncQ (15.6 kb); translational *arcD′-′lacZ* fusion	17
pME3087	Tc Mob ColE1 replicon (6.9 kb); suicide vector	52, 58
pME3087ΔE	pME3087 derivative without *Eco*RI site	This work
pME3517	Insertion of 3.2-kb *Kpn*I-*Aha*III *lacZ* fragment from pML*8* in pBluescript KS(+) cut with *Kpn*I and *Hinc*II	This work (Fig. 2A)
pME3523	Ap/Cb Km Mob IncQ (14.2 kb); insertion of 3.2-kb *Sma*I-*Sal*I *lacZ* fragment from pME3517 into pME336	This work
pME3702	pGEM-1 derivative carrying an *arcA* internal sequence	17
pME3732	pME190 derivative with deletion of 182 bp in the 3′ region of *arcD*	15
pME3781	Ap/Cb *lacZ*$^+$ Mob IncQ (13.5 kb); pKT240 derivative with artificial −40 (TTGAT....ATCAA)/−10 (TATAAT) promoter upstream of *lacZ*	54
pME3789	Ap/Cb Mob IncQ (10.0 kb); pKT240 derivative with artificial −40 (TTGAT....ATCAA)/−10 (TATAAT) promoter upstream of the multiple cloning site	This work (Fig. 3)
pME3799	Ap/Cb Mob IncQ (12.5 kb); insertion of 2.5-kb *lipAH* fragment from pSW112 in pME3789	This work (Fig. 3)
pML8	Gm Mob IncQ (16.5 kb); promoterless *lacZ* in plasmid pML10 (a derivative of pSUP104)	31
pSW112	pUC19 derivative carrying a 15.3-kb insert containing the genes *lipAH*	55

of 25 mM Tris-HCl (pH 7.4) and disrupted by sonication at 4°C. Intact cells were eliminated by pelleting at 5,000 × *g*. The supernatant was centrifuged at 100,000 × *g* for 1 h to separate soluble proteins from an insoluble pellet. The pellet containing the membrane fraction was resuspended in water. Following electrophoresis in a polyacrylamide gel, proteins were electrotransferred to a polyvinylidene difluoride membrane (Bio-Rad) in a Trans-Blot apparatus (Bio-Rad) at 4°C. The membranes were incubated in 25 mM Tris-HCl (pH 8.0)–140 mM NaCl–3 mM KCl–0.2% (wt/vol) Tween 20 with a 1:50,000 dilution of a polyclonal antilipase antiserum raised in rabbits against a synthetic peptide consisting of amino acids 48 to 62 of the N-terminal part of mature lipase (55). This peptide had been conjugated to keyhole limpet hemocyanin with glutaraldehyde as the coupling reagent. Immunoblots were treated with goat anti-rabbit horseradish peroxidase conjugate as the secondary antibody and developed with the enhanced chemiluminescence system (Amersham).

Enzyme assays. ADI and cOTC were measured in toluenized cells as described elsewhere (33). β-Galactosidase specific activities were determined by the Miller method (45). Lipase activity was measured with *p*-nitrophenylpalmitate (*p*-NPP) as the substrate (48). After incubation at 37°C for 15 min, the A_{410} was determined, with an enzyme-free control as a blank value. Under these conditions, an A_{410} of 1 equals 0.212 ng of pure lipase (48). Specific lipase activities are expressed as nanograms of lipase per 10^9 cells. Lipase activity was also determined by a specific and sensitive plate assay with the fluorescent dye rhodamine B and trioleoylglycerol (olive oil) as described previously (29).

RESULTS

Expression of a promoterless *lacZ* cartridge inserted at four different positions in the chromosomal *arcDABC* operon of *P. aeruginosa*. We chose the intrinsically unstable *lacZ* (β-galactosidase) transcripts of *E. coli* to test whether this mRNA would be stabilized when inserted into an *arc* transcript environment in *P. aeruginosa*. In *E. coli*, the chemical and functional half-lives of *lacZ* mRNA are 1 to 3 min, and nucleolytic degradation is initiated near the 5′ end of the *lacZ* transcript by RNase E (41, 53, 57). A promoterless *lacZ* cartridge was constructed in pME3517 (Fig. 2A), which contains the *E. coli lacZ* gene fused to a 66-bp fragment from bacteriophage λ and a ribosome binding site (31). Downstream of the *lacZ* gene lies a sequence whose dyad symmetry has the potential to form a hairpin structure in the transcript. This structure slightly stabilizes native *lacZ* mRNA in *E. coli* (41). The *lacZ* cartridge was inserted into the *arc* operon of *P. aeruginosa* at positions 557 (in *arcD*), 2590 (in *arcA*), and ca. 3000 (in *arcB*) (Fig. 2B). In each case, the *lacZ* insertion was made at an artificial *Eco*RI site which had been created by insertion of Tn*1725* into the *arc* operon (32). Tn*1725* contains an *Eco*RI site in each terminal inverted repeat, and in vitro excision of the interior of the transposon results in a 35-bp insertion with an *Eco*RI site (49). In addition, pME3732, a plasmid carrying a 182-bp deletion at the 3′ end of *arcD* (15), was used to construct a fourth *lacZ* fusion. Plasmid pME3732 lacks the RNase E processing sites of the *arc* operon, whereas the *arcA* translation initiation region remains functional. The *Eco*RI site marking the deletion site allowed an *arcD-lacZ* fusion to be made at position 1497 (Fig. 2B). All four *lacZ* insertions were integrated into the

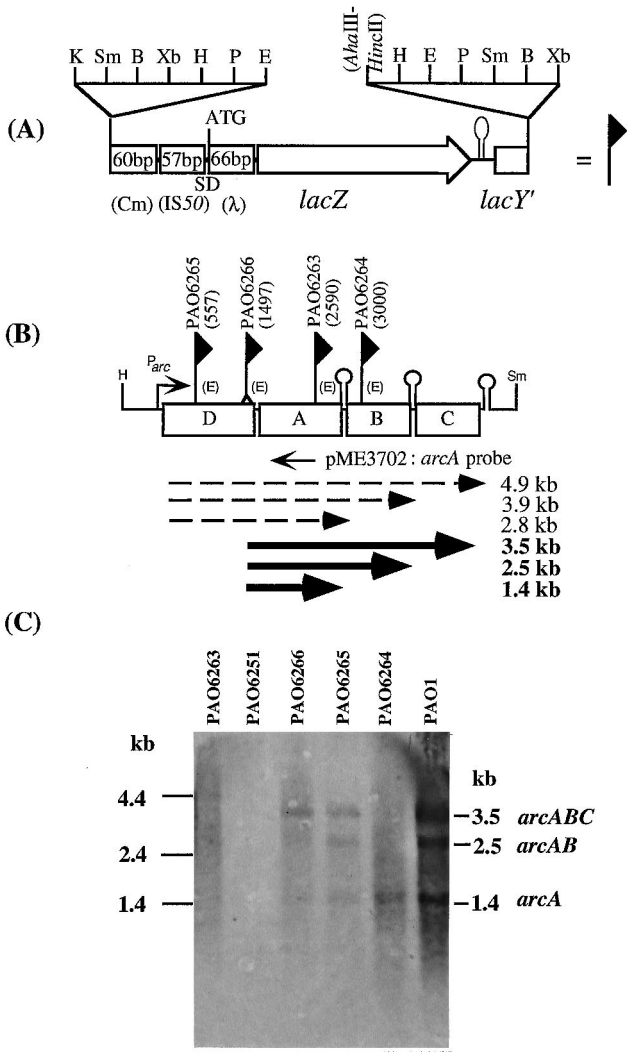

FIG. 2. Construction of chromosomal *lacZ* insertion mutants of *P. aeruginosa* and Northern blot analysis. (A) The 3.2-kb *lacZ* cartridge from pML8 (31) was cloned as a *Kpn*I-*Aha*III fragment into pBluescript KS(+) cut with *Kpn*I and *Hin*cII, resulting in pME3517, which contains the transcriptional *lacZ* probe shown. Restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Sm, *Sma*I; and Xb, *Xba*I. (B) The *lacZ* cartridge (▸) was excised as an *Eco*RI fragment and inserted into four different *Eco*RI sites in the *arc* operon giving transcriptional *arc-lacZ* fusions. These were integrated into the PAO1 chromosome, producing the mutants PAO6265 ($arcD_{557}$-*lacZ*), PAO6266 ($arcD_{1497}$-*lacZ*), PAO6263 ($arcA_{2590}$-*lacZ*), and PAO6264 ($arcB_{3000}$-*lacZ*), as described in Materials and Methods. The triangle-based flag marks the mRNA processing site(s) at the 3′ end of *arcD*, giving three stable *arc* transcripts (solid lines) derived from the primary transcripts (broken lines) (15, 16). The anaerobically inducible *arc* promoter (P_{arc}) and the direction and location of the *arcA* antisense RNA probe synthesized in vitro with T7 RNA polymerase (15) (pME3702) are indicated. *Eco*RI sites in parentheses do not exist in the natural *arc* operon. (C) Northern blot. Total RNA (PAO6263, 0.5 μg; other strains, 2 μg) was extracted from cultures grown in YEA medium with oxygen limitation for 5 h and hybridized to a DIG-labeled *arcA* probe. An RNA ladder (GIBCO-BRL) served as a size marker.

chromosomal *arc* operon of *P. aeruginosa* PAO1 via homologous recombination, using the suicide vector pME3087 (Materials and Methods). In all *lacZ* constructs, the *arc* sequences located upstream and downstream of the insertions were left intact.

The levels of β-galactosidase in cells grown with oxygen limitation were similar for the four *lacZ* strains (Table 2), indicating that the position of the *lacZ* insertion has little impact on β-galactosidase expression. We conclude that an *arcA* or *arcB* transcript environment, although stable per se, cannot enhance *lacZ* expression levels above those found in the intrinsically less stable *arcD* environment. Uninduced (aerobic) enzyme levels varied somewhat, depending on the site of *lacZ* insertion, but the effect of anaerobic induction was always apparent (Table 2). The chromosomal *lacZ* insertions in *arcD* or *arcA* had a polar effect on the expression of the downstream *arc* genes (Table 2), presumably by destabilizing the mRNA, but partial termination of transcription is also possible.

The stable, processed *arcABC*, *arcAB*, and *arcA* transcripts are not formed after *lacZ* insertion into *arcA*. The processed *arcA*, *arcAB*, and *arcABC* mRNAs can be readily detected in *P. aeruginosa* with an *arcA* probe and are much more abundant than the primary *arcDA*, *arcDAB*, and *arcDABC* transcripts, which need to be visualized by hybridization with an *arcD* probe after extensive exposure (15). To test the effect of *lacZ* insertions in the *arc* genes, we isolated RNA from oxygen-limited cultures and, following Northern transfer, revealed the transcripts by hybridization with an *arcA* probe (Fig. 2C). The typical 3.5-kb *arcABC*, 2.5-kb *arcAB*, and 1.4-kb *arcA* transcripts appeared in the wild-type PAO1 and were entirely missing in the deletion mutant PAO6251 (Δ*arcDABC*). The $arcD_{557}$-*lacZ* mutant PAO6265, which still carries the natural processing sites in *arcD*, produced the *arcABC*, *arcAB*, and *arcA* mRNAs with low efficiency. Somewhat surprisingly, the $arcD_{1497}$-*lacZ* mutant PAO6266, in which the processing region in *arcD* is deleted, also gave the same three mRNAs, albeit with low yields (Fig. 2C). Previous work had shown that in the absence of a *lacZ* insertion, the 182-bp deletion at the 3′ end of *arcD* eliminates the formation of the 3.5-, 2.5-, and 1.4-kb transcripts (15). It appears, therefore, that in strain PAO6266 the *lacZ* insert acts as an artificial processing site, with formation of some stable downstream transcripts. In the $arcA_{2590}$-*lacZ* mutant PAO6263, the discrete *arcABC*, *arcAB*, and *arcA* bands disappeared and a smear was seen, indicative of highly unstable RNA. Finally, the insertion of *lacZ* in *arcB* (PAO6264) led to a loss of the *arcABC* and *arcAB* transcripts, and only the 1.4-kb *arcA* transcript was visible (Fig. 2C). Thus, the 3.2-kb *lacZ* segment, when inserted in *arcA* or *arcB*, did not increase the length of the typical *arc* transcripts but, on the contrary, destabilized them. This result was confirmed by hybridization of the same RNA preparations with a *lacZ* probe:

TABLE 2. ADI, cOTC, and β-galactosidase levels in the wild-type PAO1 and the four *lacZ* insertion mutants

Strain	Fusion	β-Galactosidase sp act (kU)		ADI sp act[a]		cOTC sp act[a]	
		$+O_2$[b]	$-O_2$[b]	$+O_2$	$-O_2$	$+O_2$	$-O_2$
PAO1		0	0	2.3	55.4	27	1,100
PAO6263	$arcA_{2590}$-*lacZ*	0.74	3.12	0	0	76	280
PAO6264	$arcB_{ca.\ 3000}$-*lacZ*	0.24	2.28	1.4	26.2	0	0
PAO6265	$arcD_{557}$-*lacZ*	0.15	2.41	1.2	4.0	24	93
PAO6266	$arcD_{1497}$-*lacZ*	0.40	2.95	4.0	10.4	48	622

[a] ADI and cOTC specific activities are given in micromoles of product per hour per milligram of protein. The values shown are averages of three independent measurements, which differed by <20%.

[b] $+O_2$, aerobic growth: cells were grown in 500-ml baffled flasks containing 60 ml of YEA medium with vigorous shaking to exponential phase (ca. 5×10^8 cells per ml). $-O_2$, oxygen-limited growth: cells were grown in tightly capped 125-ml bottles containing 60 ml of YEA medium to stationary phase (ca. 8×10^8 cells per ml).

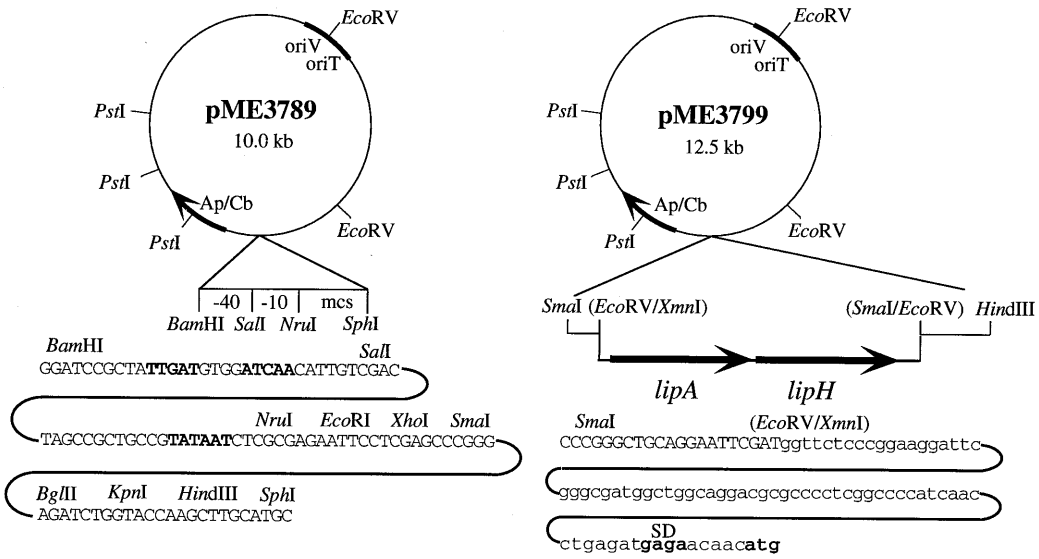

FIG. 3. Construction of the anaerobic expression vector pME3789 and its application to lipase overexpression. Plasmid pME3789 is based on the mobilizable broad-host-range IncQ vector pKT240 (2) and its derivative pME3781 (54) and carries an artificial modular promoter consisting of a −40 ANR binding site (boldface), a −10 RNA polymerase recognition sequence (boldface), and a multiple cloning site (mcs). The 2.5-kb *Xmn*I-*Sma*I *lipAH* fragment derived from pSW112 was modified at the ends (by being cloned into the *Eco*RV site of pBluescript). The resulting 2.5-kb *Sma*I-*Hin*dIII fragment was inserted into the vector pME3789, giving pME3799. The Shine-Dalgarno sequence (SD) and the *lipA* start codon are indicated in boldface.

no discrete *arc-lacZ* bands were detectable in strains PAO6263 and PAO6264 (data not shown).

Enzyme activities were determined in parallel with the RNA analysis; they agreed with the values shown in Table 2. The polar effects of *lacZ* insertion on the expression of the downstream *arc* genes can be explained adequately by the reduced amounts of *arc* transcripts (Fig. 2C).

The same *arc-lacZ* transcriptional fusions were constructed in an IncQ vector plasmid derived from pKT240. The β-galactosidase levels measured in *P. aeruginosa* (data not shown) paralleled the results obtained with the chromosomal *lacZ* fusions (Table 2), confirming that heterologous *lacZ* expression does not benefit from *lacZ* transplantation into the *arcA* and *arcB* genes. In fact, a transcriptional *lacZ* fusion made close to the *arc* promoter (at position 257 at the 5′ end of *arcD*, in plasmid pME3523) gave maximal β-galactosidase levels upon anaerobic activation (ca. 21,000 Miller units), and this observation served as the basis for the construction of optimized, ANR-dependent expression constructs.

Construction of the ANR-dependent expression vector pME3789. In previous work (17, 54), the effects of *arc* promoter mutations on oxygen-limited, ANR-dependent expression have been studied. A 20% increase of expression occurs when the −40 region GCTA **TTGAC** GTGG **ATCAG** CATT containing the Arc box (boldface) is changed to the sequence GCTA **TTGAA** GTGG **ATCAA** CATT, containing an FNR consensus inner motif (boldface). A fully symmetrical, optimal FNR binding site, AAAT **TTGAT** ATAT **ATCAA** ATTT, in contrast, is recognized by ANR rather poorly in *P. aeruginosa* (54). The −10 promoter sequence determines the levels of both aerobic and anaerobic expression. Replacement of the *arc* wild-type hexamer CCTAAT by the *E. coli* consensus hexamer TATAAT increases aerobic and anaerobic expression about threefold in *P. aeruginosa* (54). In the newly constructed IncQ vector pME3789 (Fig. 3), the −40/−10 combination giving the highest level of expression (89,000 Miller units after induction) was used, and a linker containing eight unique restriction sites was inserted downstream of the modified *arc* promoter. The copy number of pME3789 in *P. aeruginosa* is expected to be 20 to 40, as found for similar IncQ plasmids (27). Transcriptional *lacZ* fusion to the *tac* promoter, in an IncQ vector, gives ca. 47,000 Miller units after induction with isopropyl-β-D-thiogalactopyranoside (IPTG) in *P. aeruginosa* (47). Thus, the modified *arc* promoter appears to be about twice as strong as the *tac* promoter.

Application of pME3789: overexpression of *P. aeruginosa* lipase. The *lipAH* genes of *P. aeruginosa*, encoding lipase and its foldase, an export helper protein bound to the inner membrane (26, 55), were cloned on a 2.5-kb *Xmn*I-*Sma*I fragment into the vector pME3789. In the resulting construct, pME3799 (Fig. 3), the ATG start codon of the *lipA* gene lies ca. 110 bp downstream of the ANR-dependent vector promoter (Fig. 3).

The wild-type strain PAO1 grown to exponential phase with good aeration produced minute amounts of lipase in culture supernatants, whether or not the strain contained pME3799 (Table 3). Severely oxygen-limited growth in hermetically sealed vessels failed to increase extracellular lipase in cultures of strain PAO1/pME3799 (Table 3). In contrast, strain PAO1

TABLE 3. ANR-dependent expression of lipase and β-galactosidase in *P. aeruginosa*

Strain	Enzyme measured	Sp act[a]		
		$+O_2$	$-O_2$	Stat.
PAO1	Lipase	1	0	5
PAO1/pME3799 (*lipAH*$^+$)	Lipase	3	1	150
PAO1/pME3781 (*lacZ*$^+$)	β-Galactosidase	16	89	78

[a] Lipase specific activities are measured by the *p*-NPP assay (Materials and Methods) and expressed as nanograms of lipase per 10^9 cells. β-Galactosidase activities are given in thousands of Miller units. Average values of three independent experiments are shown. $+O_2$, aerobic growth in YEA medium; $-O_2$, oxygen-limited growth in YEA medium; Stat., cells grown in 500-ml baffled flasks containing 60 ml of YEA medium to ca. 8×10^8 cells per ml with aeration and then further incubated unshaken at room temperature for 40 h to stationary phase (ca. 2×10^9 cells per ml).

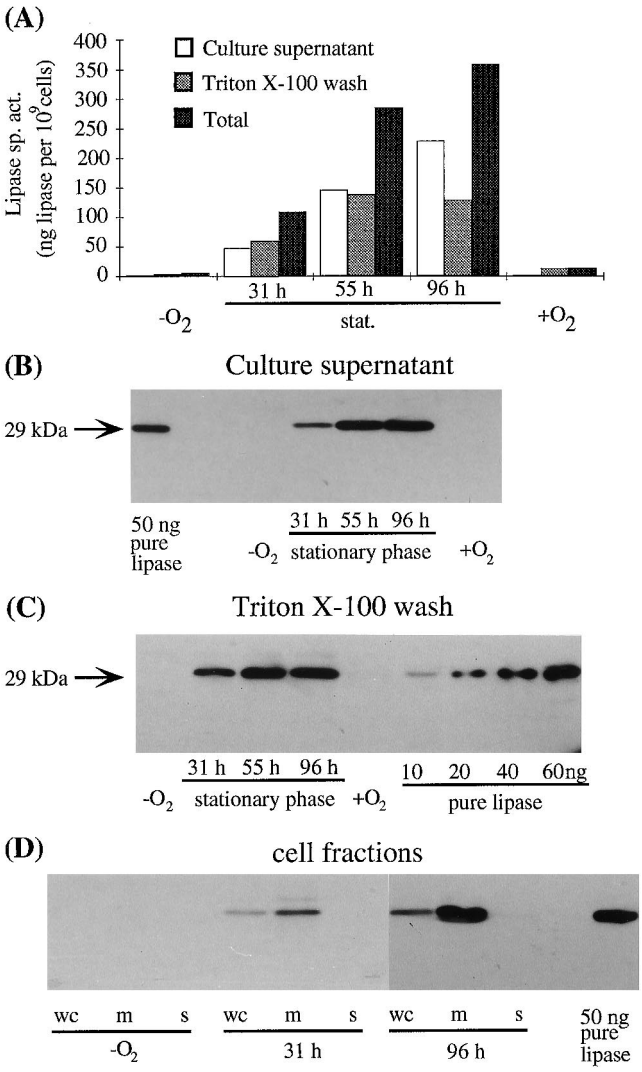

FIG. 4. Production of lipase activity and protein as a function of time. (A) Total lipase activity is composed of a cell-free supernatant fraction and a cell-associated fraction, which can be released by washing with 0.1% Triton X-100. The open, unshaken culture was incubated at room temperature for 31, 55, and 96 h. Aerobic (+O_2) and anaerobic (−O_2) incubations were done as described in Table 2, footnote *b*. stat., stationary phase; sp. act., specific activity. (B) Dialyzed cell-free supernatants from culture samples containing 3.2×10^8 cells were concentrated by evaporation under reduced pressure and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblot analysis using antilipase antibodies. (C) Cells were washed with 0.1% Triton X-100. The wash solution, containing lipase released from the cells, was dialyzed and subjected to SDS-PAGE followed by immunoblot analysis. The same amounts of supernatants as in panel B were loaded. Increasing amounts of pure lipase (10, 20, 40, and 60 ng) were run in order to quantify the amount of lipase produced. (D) Samples of 10 μg of total protein of whole cells (wc), the membrane fraction (m), and the soluble fraction (s) of anaerobic and stationary (31 and 96-h) cultures were subjected to SDS-PAGE followed by immunoblot analysis.

harboring pME3781, an analogous, ANR-dependent plasmid expressing the *lacZ* gene of *E. coli* (54), gave very high levels of intracellular β-galactosidase upon oxygen limitation in closed bottles (Table 3). Here, β-galactosidase represented about 20% of the total soluble cell protein. Thus, these culture conditions turn on the vector promoter but are not conducive to overproduction of extracellular lipase. Earlier work (8) has shown that in *P. aeruginosa* MB5001, lipase production is optimal when the cultures are shifted from oxygen limitation to oxygen-nonlimiting conditions in the stationary growth phase. A possible interpretation is that the presence of some oxygen is required for secretion of active lipase. Therefore, strain PAO1/pME3799 was grown to ca. 8×10^8 cells per ml with shaking and then incubated as an open, unshaken culture at room temperature for 1 day or longer. In such cells, the ANR-dependent promoter is turned on, as shown by high-level β-galactosidase expression in strain PAO1/pME3781 (Table 3). However, atmospheric oxygen is available to the cells. Strain PAO1/pME3799 cultivated in this way produced 150 ng of lipase per 10^9 cells after 40 h of incubation, a 30-fold increase compared with lipase production by strain PAO1 (Table 3).

Lipase production in static cultures of strain PAO1/pME3799 was monitored as a function of time. Over a 4-day period, there was an increase of lipase activity and protein in the culture supernatants (Fig. 4A and B), and additional lipase could be recovered by washing the cells with Triton X-100 (Fig. 4A and C). Cell-associated lipase was found in the insoluble (membrane) fraction and could not be detected among soluble proteins. Processed 29-kDa lipase was observed in all fractions, and only a minor portion of the membrane-associated enzyme appeared as a 33-kDa band typical of unprocessed lipase (Fig. 4D). Cells grown with good aeration or under strict oxygen limitation did not produce intra- or extracellular lipase protein (Fig. 4B and D). In particular, severely oxygen-limited cells did not accumulate any lipase protein in the cytoplasm or in the membrane fraction, indicating that lack of oxygen interferes with lipase synthesis or secretion.

DISCUSSION

Development of an expression vector derived from the *arc* operon of *P. aeruginosa*. The existence of mRNA stabilizers in *E. coli* initially encouraged us to test whether the remarkable stability of the processed *arcA*, *arcAB*, and *arcABC* transcripts of *P. aeruginosa* could be exploited to stabilize heterologous, unstable mRNAs. Stem-loop structures positioned at the 3′ ends of transcripts can function as barriers to 3′-to-5′ mRNA degradation and enhance mRNA stability (5, 42, 56). For instance, when the 3′ element of dyad symmetry of the *lacZ* gene of *E. coli* is deleted (Fig. 2A), the functional half-life of *lacZ* mRNA can be decreased up to threefold (41). In the *arc* operon of *P. aeruginosa* (Fig. 1), the inverted repeats IR_1 and IR_2 have a mild mRNA-stabilizing effect (15). The 5′ ends of bacterial mRNAs can also have a crucial impact on stability. For instance, *ompA* mRNA in *E. coli* owes much of its longevity to the 5′ untranslated region. A particular fusion of this region to the *lacZ* gene enhances the chemical half-life of *lacZ* mRNA fivefold (22). However, it appears that the fusion sites in *ompA* and *lacZ* are important, since in other experiments with different *ompA-lacZ* fusions no stabilizing effect has been detected (18).

The stability of many different mRNAs of *E. coli*, including that of *lacZ* mRNA, critically depends on RNase E, an endonuclease which cleaves transcripts at specific sites, often near the 5′ end (12, 21, 37, 57). RNase E has been found in a protein complex which additionally contains polynucleotide phosphorylase (7, 43). The latter enzyme, together with RNase II, exonucleolytically degrades the bulk of mRNA in the 3′-to-5′ direction (5, 42). There is evidence that *P. aeruginosa* contains an RNase E-like enzyme: the *arc* transcripts are processed at the same (or similar) sites in *P. aeruginosa* and *E. coli* (16). In a temperature-sensitive RNase E mutant of *E. coli*, at nonpermissive temperature, the *arc* transcripts are not processed (16). In *P. aeruginosa*, deletion of the processing site(s) in *arcD* has

the same effect (15). For this organism, however, no RNase E mutants are available.

Insertion of *lacZ* into *arcA* abolished the typical *arcA*, *arcAB*, and *arcABC* transcripts, rather than making them longer (Fig. 2C). This indicates that *lacZ* mRNA is a highly vulnerable endonuclease target not only in *E. coli* (41) but also in *P. aeruginosa*. Integration of *lacZ* into *arcB* destabilized the *arcAB* and *arcABC* mRNAs but spared *arcA* mRNA (Fig. 2C), suggesting that *lacZ* insertion induces an overall 5′-to-3′ degradation, as seen for *lacZ* mRNA in *E. coli* (35). The most labile part of *lacZ* mRNA in *E. coli* resides in the ribosome binding-translation initiation sites (53), in which decay is initiated by RNase E (57). It is plausible that the RNase E-like enzyme of *P. aeruginosa* may play an analogous role. Evidence comes from strain PAO6266 ($arcD_{1497}$-*lacZ*), in which the natural *arcD* processing region is deleted: here, the *lacZ* insert appears to act as a substitute processing site (Fig. 2C).

Since promoter-proximal *lacZ* insertions in plasmid constructs gave the highest β-galactosidase levels, we focused on the promoter region for vector construction. Modifications of the −40 and −10 regions of the native *arc* promoter increased oxygen-limited expression about fourfold, as judged from β-galactosidase levels. The modified promoter, by virtue of its −10 consensus hexamer TATAAT, also gave fairly high noninduced β-galactosidase values (Table 3). When this is undesirable, the modular construction of pME3789 permits a straightforward replacement of the −10 region (Fig. 3). For instance, the *arc* −10 hexamer CCTAAT, in combination with various ANR binding sites, results in lower induced and noninduced levels as well as in a tighter control by oxygen limitation (54).

Applications of the ANR-dependent expression system. *Pseudomonas* lipases are of biotechnological interest, primarily because of their stereospecificity (8, 26). As heterologous expression of lipase can pose problems of secretion, production of this enzyme is usually sought in the original host. To our knowledge, convenient expression vectors that allow increased lipase production in *Pseudomonas* species have not been reported, and industrial lipase production processes appear to rely mostly on conventional strain improvement and optimization of culture conditions (8, 26, 34). Using the pME3789-*lipAH* expression construct, we demonstrate that in wild-type *P. aeruginosa* PAO1, lipase overproduction can be achieved in a simple fermentation process (Fig. 4). No attempt to optimize the culture medium and the dissolved-oxygen tension during the incubation was made.

FNR-dependent, oxygen-controlled promoters have been used in *E. coli* and *Salmonella typhimurium* to overproduce a range of proteins of industrial interest, such as antigens for vaccine purposes (9, 38, 40). Our present study illustrates that a similar approach based on ANR-dependent expression is successful in *P. aeruginosa*. To some extent, FNR- and ANR-dependent systems are interchangeable in that FNR and ANR have overlapping recognition specificities. However, there are limits to such interchange. For instance, the *P. aeruginosa arc* promoter does not function in fnr^+ *E. coli* (33, 54). In contrast, the *arc* promoter is functional and inducible in several species of fluorescent pseudomonads (59). This opens up the possibility of using ANR-dependent vectors in *Pseudomonas* species other than *P. aeruginosa.*

ACKNOWLEDGMENTS

We thank SmithKline Beecham for a generous gift of carbenicillin.

This project was supported by a grant of the Eidgenössische Technische Hochschule Zürich during the initial phase and by EU project BIO4-CT96-0119. B.S. acknowledges support from the Deutsche Forschungsgemeinschaft.

REFERENCES

1. **Akrim, M., M. Bally, G. Ball, J. Tommassen, H. Teerink, A. Filloux, and A. Lazdunski.** 1993. Xcp-mediated protein secretion in *Pseudomonas aeruginosa*: identification of two additional genes and evidence for regulation of *xcp* gene expression. Mol. Microbiol. **10:**431–443.
2. **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.
3. **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.
4. **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.
5. **Belasco, J. G., and C. F. Higgins.** 1988. Mechanisms of mRNA decay in bacteria: a perspective. Gene **72:**15–23.
6. **Bourdineaud, J.-P., D. Heierli, M. Gamper, H. J. C. Verhoogt, A. J. M. Driessen, W. N. Konings, C. Lazdunski, and D. Haas.** 1993. Characterization of the *arcD* arginine:ornithine exchanger of *Pseudomonas aeruginosa*. J. Biol. Chem. **268:**5417–5424.
7. **Carpousis, A. J., G. Van Houwe, C. Ehretsmann, and H. M. Krisch.** 1994. Copurification of *E. coli* RNase E and PNPase: evidence for a specific association between two enzymes important in RNA processing and degradation. Cell **76:**889–900.
8. **Chartrain, M., C. Marcin, L. Katz, P. Salmon, T. Brix, B. Buckland, and R. Greasham.** 1993. Enhancement of lipase production during fed-batch cultivation of *Pseudomonas aeruginosa* MB 5001. J. Ferm. Bioeng. **76:**487–492.
9. **Chatfield, S. N., I. G. Charles, A. J. Makoff, M. D. Oxer, G. Dougan, D. Pickard, D. Slater, and N. F. Fairweather.** 1992. Use of the *nirB* promoter to direct the stable expression of heterologous antigens in *Salmonella* oral vaccine strains: development of a single-dose oral tetanus vaccine. Bio/Technology **10:**888–892.
10. **Cohen, S. N.** 1995. Surprises at the 3′ end of prokaryotic RNA. Cell **80:**829–832.
11. **Del Sal, G., G. Manfioletti, and C. Schneider.** 1988. A one-tube plasmid DNA mini-preparation suitable for sequencing. Nucleic Acids Res. **16:**9878.
12. **Faubladier, M., K. Cam, and J.-P. Bouché.** 1990. *Escherichia coli* cell division inhibitor DicF-RNA of the *dicB* operon. Evidence for its generation *in vivo* by transcription termination and by RNase III and RNase E-dependent processing. J. Mol. Biol. **212:**461–471.
13. **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.
14. **Gamper, M.** 1991. Regulation des *arc*-Operons von *Pseudomonas aeruginosa*. Ph.D. thesis no. 9415. Eidgenössische Technische Hochschule, Zürich.
15. **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.
16. **Gamper, M., and D. Haas.** 1993. Processing of the *Pseudomonas arcDABC* mRNA requires functional RNase E in *Escherichia coli*. Gene **129:**119–122.
17. **Gamper, M., A. Zimmermann, and D. Haas.** 1991. Anaerobic regulation of transcription initiation in the *arcDABC* operon of *Pseudomonas aeruginosa*. J. Bacteriol. **173:**4742–4750.
18. **Green, P. J., and M. Inouye.** 1984. Roles of the 5′ leader region of the *ompA* mRNA. J. Mol. Biol. **176:**431–442.
19. **Haas, D., M. Gamper, and A. Zimmermann.** 1992. Anaerobic control in *Pseudomonas aeruginosa*, p. 177–187. *In* S. Silver, E. Galli, and B. Witholt (ed.), *Pseudomonas*: molecular biology and biotechnology. American Society for Microbiology, Washington, D.C.
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. **Hajnsdorf, E., O. Steier, L. Coscoy, L. Teysset, and P. Régnier.** 1994. Roles of RNase E, RNase II and PNPase in the degradation of the *rpsO* transcripts of *Escherichia coli*: stabilizing function of RNase II and evidence for efficient degradation in an *ams pnp rnb* mutant. EMBO J. **13:**3368–3377.
22. **Hansen, M. J., L.-H. Chen, M. L. S. Fejzo, and J. G. Belasco.** 1994. The *ompA* 5′ untranslated region impedes a major pathway for mRNA degradation in *Escherichia coli*. Mol. Microbiol. **12:**707–716.
23. **Higgins, C. F., H. C. Causton, G. S. C. Dance, and E. A. Mudd.** 1993. The role of the 3′ end in mRNA stability and decay, p. 13–30. *In* J. Belasco and G. Brawerman (ed.), Control of messenger RNA stability. Academic Press, Inc., San Diego, Calif.
24. **Holloway, B. W., U. Römling, and B. Tümmler.** 1994. Genomic mapping of *Pseudomonas aeruginosa* PAO. Microbiology **140:**2907–2929.
25. **Jaeger, K.-E., S. Ransac, B. W. Dijkstra, C. Colson, M. van Heuvel, and O. Misset.** 1994. Bacterial lipases. FEMS Microbiol. Rev. **15:**29–63.
26. **Jaeger, K.-E., B. Schneidinger, K. Liebeton, D. Haas, M. T. Reetz, S. Philippou, G. Gerritse, S. Ransac, and B. W. Dijkstra.** Lipase of *Pseudomonas aeruginosa*: molecular biology and biotechnological application. *In* T. Nakazawa, K. Furukawa, D. Haas, and S. Silver (ed.), *Pseudomonas*: Molecular

biology and biotechnology, in press. American Society for Microbiology, Washington, D.C.
27. **Jeenes, D. J., L. Soldati, H. Baur, J. M. Watson, A. Mercenier, C. Reimmann, T. Leisinger, and D. Haas.** 1986. Expression of biosynthetic genes from *Pseudomonas aeruginosa* and *Escherichia coli* in the heterologous host. Mol. Gen. Genet. **203:**421–429.
28. **Khosla, C., J. E. Curtis, P. Bydalek, J. R. Swartz, and J. E. Bailey.** 1990. Expression of recombinant proteins in *Escherichia coli* using an oxygen-responsive promoter. Bio/Technology **8:**554–558.
29. **Kouker, G., and K.-E. Jaeger.** 1987. Specific and sensitive plate assay for bacterial lipases. Appl. Environ. Microbiol. **53:**211–213.
30. **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.
31. **Labes, M., A. Pühler, and R. Simon.** 1990. A new family of RSF1010-derived expression and *lac*-fusion broad-host-range vectors for Gram-negative bacteria. Gene **89:**37–46.
32. **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.
33. **Lüthi, E., A. Mercenier, and D. Haas.** 1986. The *arcABC* operon required for fermentative growth of *Pseudomonas aeruginosa* on arginine: Tn*5-75l*-assisted cloning and localization of structural genes. J. Gen. Microbiol. **132:** 2667–2675.
34. **Marcin, C., L. Katz, R. Greasham, and M. Chartrain.** 1993. Optimization of lipase production by *Pseudomonas aeruginosa* MB 5001 in batch cultivation. J. Ind. Microbiol. **12:**29–34.
35. **McCormick, J. R., J. M. Zengel, and L. Lindahl.** 1994. Correlation of translational efficiency with the decay of *lacZ* mRNA in *Escherichia coli*. J. Mol. Biol. **239:**608–622.
36. **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.
37. **Mudd, E. A., H. M. Krisch, and C. F. Higgins.** 1990. RNase E, an endoribonuclease, has a general role in the chemical decay of *Escherichia coli* mRNA: evidence that *rne* and *ams* are the same genetic locus. Mol. Microbiol. **4:**2127–2135.
38. **Newton, S. M. C., P. E. Klebba, M. Hofnung, and A. Charbit.** 1995. Studies of the anaerobically induced promoter p*nirB* and the improved expression of bacterial antigens. Res. Microbiol. **146:**193–202.
39. **Oshima-Hirayama, N., K. Yoshikawa, T. Nishioka, and J. Oda.** 1993. Lipase from *Pseudomonas aeruginosa*. Production in *Escherichia coli* and activation *in vitro* with a protein from the downstream gene. Eur. J. Biochem. **215:**239–246.
40. **Oxer, M. D., C. M. Bentley, J. G. Doyle, T. C. Peakman, I. G. Charles, and A. J. Makoff.** 1991. High level heterologous expression in *E. coli* using the anaerobically-activated *nirB* promoter. Nucleic Acids Res. **19:**2889–2892.
41. **Petersen, C.** 1991. Multiple determinants of functional mRNA stability: sequence alterations at either end of the *lacZ* gene affect the rate of mRNA inactivation. J. Bacteriol. **173:**2167–2172.
42. **Petersen, C.** 1992. Control of functional mRNA stability in bacteria: multiple mechanisms of nucleolytic and non-nucleolytic inactivation. Mol. Microbiol. **6:**277–282.
43. **Py, B., H. Causton, E. A. Mudd, and C. F. Higgins.** 1994. A protein complex mediating mRNA degradation in *Escherichia coli*. Mol. Microbiol. **14:**717–729.
44. **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.
45. **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.
46. **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.
47. **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.
48. **Stuer, W., K.-E. Jaeger, and U. K. Winkler.** 1986. Purification of extracellular lipase from *Pseudomonas aeruginosa*. J. Bacteriol. **168:**1070–1074.
49. **Ubben, D., and R. Schmitt.** 1986. Tn*1725* derivatives for transposon mutagenesis, restriction mapping and nucleotide sequence analysis. Gene **41:** 145–152.
50. **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.
51. **Verhoogt, H. J. C., H. Smit, T. Abee, M. Gamper, A. J. M. Driessen, D. Haas, and W. N. Konings.** 1992. *arcD*, the first gene of the *arc* operon for anaerobic arginine catabolism in *Pseudomonas aeruginosa*, encodes an arginine-ornithine exchanger. J. Bacteriol. **174:**1568–1573.
52. **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 Publishers, Weinheim, Germany.
53. **Wagner, L. A., R. F. Gesteland, T. J. Dayhuff, and R. B. Weiss.** 1994. An efficient Shine-Dalgarno sequence but not translation is necessary for *lacZ* mRNA stability in *Escherichia coli*. J. Bacteriol. **176:**1683–1688.
54. **Winteler, H., 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.
55. **Wohlfarth, S., C. Hoesche, C. Strunk, and U. K. Winkler.** 1992. Molecular genetics of the extracellular lipase of *Pseudomonas aeruginosa* PAO1. J. Gen. Microbiol. **138:**1325–1335.
56. **Wong, H. C., and S. Chang.** 1986. Identification of a positive retroregulator that stabilizes mRNAs in bacteria. Proc. Natl. Acad. Sci. USA **83:**3233–3237.
57. **Yarchuk, O., N. Jacques, J. Guillerez, and M. Dreyfus.** 1992. Interdependence of translation, transcription and mRNA degradation in the *lacZ* gene. J. Mol. Biol. **226:**581–596.
58. **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.
59. **Zimmermann, 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.