

Transcriptional Analysis of the Amidase Operon from *Pseudomonas aeruginosa*

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The transcriptional start point for the amidase structural gene (*amiE*) of *Pseudomonas aeruginosa* has been identified, and the promoter (pE) has been shown to function constitutively, as predicted for a system regulated by transcription antitermination. Northern (RNA) analysis results show that in cells grown under inducing conditions, a major 1.3-kb *amiE* transcript arises from pE, and in addition, a larger transcript of approximately 5.0 kb in length has been shown to derive from the same promoter, encoding all of the genes of the operon. DNA sequencing and S1 nuclease mapping have located a transcription terminator downstream of *amiE*, which terminates approximately half of the pE transcripts. Previously, two RpoN-dependent promoter-like sequences (pN1 and pN2) were identified upstream of the negative regulator gene, *amiC*, and we have now constructed a promoter probe vector which shows weak constitutive promoter activity within this region. This promoter would be expected to provide basal levels of expression of the *amiC* and *amiR* regulatory genes to allow induction of the system.

The amidase enzyme (EC 3.5.1.4) of *Pseudomonas aeruginosa* is produced in response to short-chain aliphatic amides, such as acetamide, in the growth medium (2, 13). Regulation of amidase (*amiE*) expression is determined by at least two separate control circuits. A positive control circuit is mediated by the product of the *amiR* gene, and expression is also subject to catabolite repression by succinate and, thus, regulation by the global catabolite repression system of *P. aeruginosa* (12, 15, 20). The amidase genes from the wild-type strain, PAC1, and an up-promoter constitutive mutant, PAC433, have been cloned into pBR322 on *Hind*III-*Sal*I DNA fragments to generate plasmids pAS20 and pJB950, respectively (5, 9, 22).

The organization of the genes of the operon is shown in Fig. 1A. The *amiE* structural gene lies closest to the proposed promoter region (1). The *amiR* gene, which lies 2 kb downstream from *amiE* and is transcribed in the same direction, was subsequently identified and sequenced (6, 14). DNA sequencing studies of the *amiE* leader region identified a consensus *Escherichia coli* σ 70 promoter sequence, a short open reading frame of unknown function, and a rho-independent transcription terminator, followed by the *amiE* gene (Fig. 1B). Under inducing growth conditions, AmiR is thought to allow RNA polymerase to read through this terminator and into the operon by a transcription antitermination mechanism (10).

A second regulatory gene, *amiC*, which negatively regulates amidase expression was then identified (22). Mutations in *amiC* thus lead to constitutive amidase expression. AmiC exerts its regulatory role by a posttranslational interaction with AmiR, and inducible amidase expression can be reconstituted in *E. coli* with the *amiECR* genes alone. Initial amino acid sequence analysis (23) and the recently solved crystal structure of AmiC (16) show that despite its cytoplasmic location, AmiC is related to the structural family of periplasmic binding proteins. Equilibrium dialysis studies have shown the binding of both inducing and noninducing amides to AmiC (23), and the binding of inducing amides is thought to relieve AmiC repres-

sion of AmiR by an uncharacterized mechanism. Two additional genes, *amiB* and *amiS*, make up the amidase operon. From deduced amino acid sequence homology searches, these two genes are thought to form part of an amide transport system (11).

Initial studies of the *amiR* gene region showed that there was promoter activity some distance upstream of the gene, and subsequently two adjacent RpoN-dependent promoter-like sequences were identified immediately upstream of *amiC* by sequence homology (6, 22). No other promoter sequences have been identified within the operon. In this paper, we report the transcriptional organization of the amidase operon of *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* and *P. aeruginosa* strains and parental and newly constructed plasmids used in this study are listed in Table 1.

Media. *E. coli* strains were grown at 37°C in Lennox broth, and *P. aeruginosa* strains were grown at 37°C in Oxoid no. 2 broth. For RNA isolations, strains were grown in *E. coli* minimal medium or *Pseudomonas* basal medium as described previously (8), with succinate as the carbon source for *E. coli* and lactate as the carbon source for *P. aeruginosa*. For amidase assays, cells were grown as described previously (8).

Amidase assays. Amidase activity in intact cells was determined by the transferase assay (2) with acetamide as the substrate. Activity levels presented in this article are the mean values of duplicate assays carried out on at least three separate occasions. One unit represents 1 μ mol of acetohydroxamate formed per min. Specific activities are expressed as units per milligram of bacterial cells (8).

RNA isolation. RNA was isolated by a modification of the method described in reference 7. A bacterial culture (500 ml) was grown to mid-exponential phase of growth. The cells were pelleted by centrifugation in a Sorvall RC2-B at 7,000 $\times g$ for 10 min at 4°C, washed with 5 ml of 50 mM Tris-HCl (pH 7.0), and recentrifuged. The cells were then resuspended in 15 ml of prewarmed lysis buffer (50 mM Tris-HCl [pH 7.0], 4% sodium dodecyl sulfate, 6% phenol) and incubated at 56°C for 5 min. Solid cesium chloride (5 g) was added to the lysate, and the solution was mixed to aid solubilization. The cell debris was removed by centrifugation as described for cell pelleting, and the clear supernatant was layered over a 2.5-ml cushion of 5.7 M cesium chloride in a 13.2-ml Beckman Ultraclear open top tube (14 by 89 mm). The sample was centrifuged in a Beckman SW41Ti rotor in an L7 ultracentrifuge at 40,000 rpm for 20 h at 20°C. The resulting RNA pellets were retrieved and resuspended in diethylpyrocarbonate-treated water. RNA was quantified spectrophotometrically.

Northern (RNA) blotting. RNA samples were analyzed on 1% formaldehyde gels prepared as described previously (17). An RNA ladder (Bethesda Research Laboratories) was used as a size marker. The RNA was transferred from the gel to a nylon membrane (Biodyne A; Pall) by the capillary blot technique (18). Filters were prehybridized, hybridized, and washed as described previously (18).

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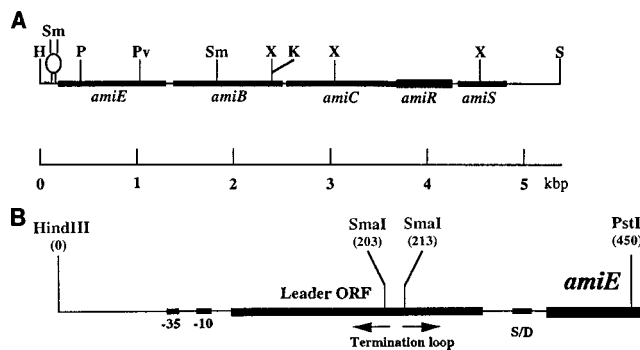


FIG. 1. Amidase operon of *P. aeruginosa*. (A) Gene organization of amidase operon. Restriction sites are H (*Hind*III), Sm (*Sma*I), P (*Pst*I), Pv (*Pvu*II), X (*Xho*I), K (*Kpn*I), and S (*Sal*I). (B) Amidase operon leader region. The locations of the σ 70 promoter sequences, the leader open reading frame (ORF), the rho-independent transcription terminator, the *amiE* Shine-Dalgarno (S/D) sequence, and the beginning of the *amiE* open reading frame are shown.

DNA labelling. Oligonucleotides were 5' end labelled as described previously (18). Uniformly labelled DNA probes were prepared for Northern analysis using the Boehringer Mannheim random-primed DNA-labelling kit and [α - 32 P]dCTP (3,000 Ci/mmol). DNA (25 to 100 ng) was labelled following the manufacturer's instructions. Unincorporated nucleotides were removed by gel filtration through Sephadex G-50. Double-stranded probes were denatured immediately prior to use by the addition of 0.1 N NaOH and incubation at 37°C for 5 min.

Primer extensions. Primer extension analysis was carried out by using end-labelled oligonucleotides. RNA was hybridized to the oligonucleotide by incubation in hybridization buffer (0.25 M KCl, 10 mM Tris-HCl [pH 8.3]). The solution was heated to 85°C for 5 min, incubated at 57°C for 1 h, and then subjected to ethanol precipitation. The hybridized RNA-DNA pellet was resuspended in 3 μ l of H₂O, and then 10 μ l of 2 \times reaction buffer (80 mM Tris-HCl [pH 8.3], 80 mM KCl, 16 mM MgCl₂, 5 μ l of a 2 mM solution of all four deoxynucleotide triphosphates, 1 μ l (25 U) of placental RNase inhibitor (Promega), and 1 μ l of avian myeloblastosis virus reverse transcriptase (Anglian Biotechnology) were added. The reaction mixture was incubated for 1 h at 50°C. Extension products were recovered by ethanol precipitation and analyzed on 8% polyacrylamide-urea sequencing gels.

S1 nuclease mapping. Single-stranded continuously labelled probes were prepared from appropriate M13 templates and used in S1 nuclease-mapping experiments as described previously (3).

DNA sequencing. The 156-bp *Nae*I fragment from pAS20 which includes the 3' end of the *amiE* gene and the 5' end of the *amiB* gene was isolated from an agarose gel and blunt end cloned into *Sma*I-cut M13mp18 as described previously (22). Recombinants with the insert in both orientations were identified, and single-stranded templates for sequencing were prepared by standard methods.

DNA sequences were determined by the chain termination method (19) with a universal primer, standard and 7-deaza-dGTP premixes (Pharmacia), and 35 S-dATP (NEN).

RESULTS

Analysis of *amiE* promoter. Previous studies have identified a DNA sequence homologous to the consensus *E. coli* σ 70 promoter some distance upstream of the *amiE* gene (Fig. 2A). This promoter was believed to function constitutively such that regulated amidase expression arose from AmiR-mediated antitermination at the transcription terminator located between the promoter and the *amiE* gene (10). Primer extension analysis has been used to identify the transcription start point for the *amiE* promoter. RNA was isolated from noninduced and induced cultures of *P. aeruginosa* PAC1, *E. coli* pJB950 (constitutive amidase genes cloned in pBR322 [5]), and *P. aeruginosa* PAC452 pSW101. PAC452 has an amidase chromosomal deletion, and pSW101 is a broad-host-range plasmid carrying the wild-type inducible amidase gene fragment (*Hind*III-*Sal*I), which shows high levels of inducible amidase activity (22). Primer extension analysis was carried out with these RNAs by using an oligonucleotide complementary to a region upstream of the transcription terminator (Fig. 2A). The results are shown in Fig. 2B. In all of the samples, the transcription start point maps to position 129, confirming that the *E. coli*-like promoter is used (Fig. 2A). In addition, the analysis of PAC1 induced and noninduced RNAs confirms that the promoter is constitutive since similar amounts of primer extension product are produced irrespective of amidase induction.

Interestingly, the amount of the primer extension product produced by pSW101, with a copy number of approximately 13 in *P. aeruginosa*, is much larger than that produced by pJB950 (copy number of 25 to 30) in *E. coli*. This is despite the fact that the *amiE* promoter has only one mismatch with the consensus *E. coli* promoter and suggests that there could be other factors enhancing transcription in *P. aeruginosa* that are not present in *E. coli*. The *amiE* promoter will be called pE.

Northern analysis. DNA sequencing of the *amiB* and *amiS* genes failed to reveal any promoter-like sequences from which these genes could be expressed. Indeed, there appeared to be a potential transcription terminator sequence between the *amiE* and *amiB* genes (21). To identify how these genes were

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description or relevant feature(s)	Reference or source
Strains		
<i>E. coli</i> JA221	<i>hsdR recA trp leu</i>	4
<i>P. aeruginosa</i>		
PAC1	Inducible Ami ⁺ prototroph	13
PAC452	<i>ami</i> 161, Ami deletion	M. Day
Plasmids		
pBR322-derived		
pJB950	Ap ^r amidase operon from PAC433 (<i>Hind</i> III- <i>Sal</i> I)	5
pAS20	Ap ^r amidase operon from PAC1 (<i>Hind</i> III- <i>Sal</i> I)	22
pSW5	Ap ^r pJB950 derivative, deletion of 658-bp <i>Xho</i> I fragment	22
pSW9	Ap ^r pBR322 carrying multiple cloning site from pUC18	This work
pSW10	Ap ^r pSW9 with promoterless <i>amiE</i> gene	This work
pSW11	Ap ^r pSW10 with 658-bp <i>Xho</i> I pAS20 fragment (wrong way)	This work
pSW12	Ap ^r pSW10 with 658-bp <i>Xho</i> I pAS20 fragment (right way)	This work
Other plasmid-derived		
pSW101	Sm ^r pKT231 derivative carrying amidase operon from PAC1	21

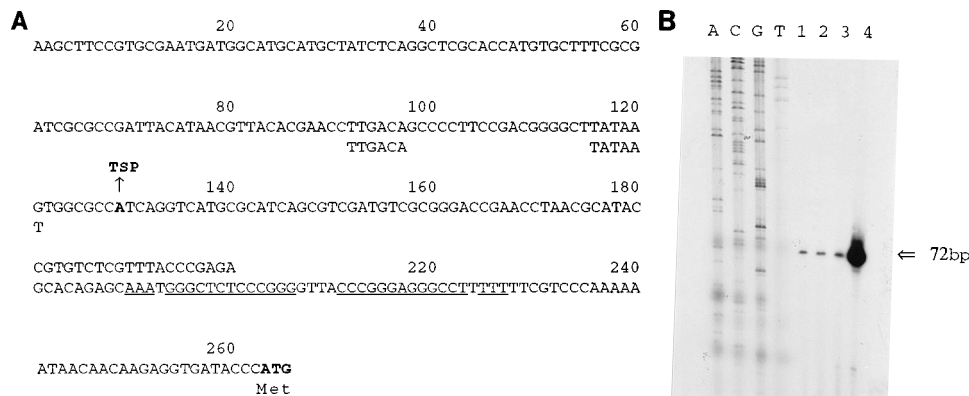


FIG. 2. Transcription analysis of *amiE* promoter. (A) DNA sequence of *amiE* leader region. The sequence of the *E. coli* consensus promoter is shown below (residues 92 to 97 [-35] and 116 to 121 [-10]). The transcription start point (TSP) at position 129 is shown in bold. The oligonucleotide used in the primer extension studies is shown above its complementary sequence (residues 181 to 200). The location of the transcription terminator is underlined, and the *amiE* start codon is shown in bold. (This figure is redrawn from reference 10.) (B) Primer extension analysis of *amiE* transcription start point. Lanes: A, C, G, and T, DNA sequencing tracks; 1 to 4, primer extensions with RNA isolated from noninduced PAC1, lactamide-induced PAC1, *E. coli* JA221 pJB950, and lactamide-induced PAC452 pSW101, respectively.

transcribed and to investigate the transcription of the other amidase genes, Northern blot analysis was carried out.

Initially, Northern blots were probed with an *amiE*-specific probe, and the results are shown in Fig. 3A. A 4-h exposure of the blot revealed the presence of a major band of 1.3 kb (lanes 1 and 2), corresponding to transcripts originating from the *amiE* promoter and terminating at the inverted repeat sequence between *amiE* and *amiB* (Fig. 4B). In Fig. 3A, lane 1 contains RNA isolated from JA221 pJB950, and lane 2 contains RNA from induced PAC1. However, in lane 3, which contains RNA isolated from induced PAC452 pSW101, the 1.3-kb *amiE* band, although present, is obscured by a broad smear, which extends up to 5.0 kb in length. The nature of this smear was initially unclear. Overnight exposures of the blot clearly revealed transcripts larger than 1.3 kb in lanes 1 and 2, which appeared to be less abundant than the 1.3-kb transcript and relatively unstable since no discrete bands were observed. This analysis suggested that these larger transcripts originated from the *amiE* promoter and resulted from read-through of a transcription terminator downstream of *amiE*.

To confirm that this was the case, Northern blots were probed with a series of different probes which extended over each downstream gene of the amidase operon. Each of these

probes gave rise to a similar transcript pattern, that is a smear extending from approximately 5.0 to 0.24 kb, with interruptions in the smear at the points where the rRNAs appear on the blots. The transcript pattern for an *amiCR*-specific probe is shown in Fig. 3B. This probe highlights similar smears from JA221 pJB950 (lane 1), induced PAC1 (lane 2), noninduced PAC452 pSW101 (lane 3), and lactamide-induced PAC452 pSW101. None of the probes from downstream of *amiE* hy-

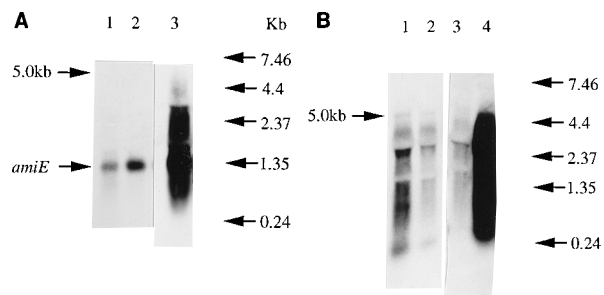


FIG. 3. Northern analysis of amidase genes. (A) RNA was isolated from JA221 pJB950 (lane 1), induced PAC1 (lane 2), and induced PAC452 pSW101 (lane 3), and the blot was probed with a *Pst*I-*Pvu*II *amiE* gene fragment. (B) RNA was isolated from JA221 pJB950 (lane 1), induced PAC1 (lane 2), noninduced PAC452 pSW101 (lane 3), and induced PAC452 pSW101 (lane 4) and probed with the 1.5-kb *Xho*I *amiCR* gene fragment. Molecular size markers are indicated on the right.

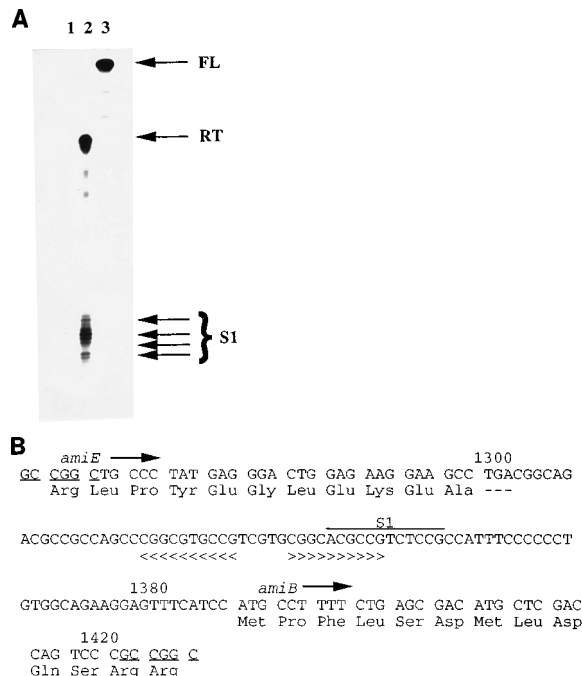


FIG. 4. Investigation of *amiE* downstream terminator. (A) S1 mapping of 3' end of *amiE* transcript, by using a continuously labeled *Nae*I(1268)-*Nae*I(1423) single-stranded probe. Lanes: 1, noninduced PAC452 pSW101; 2, lactamide-induced PAC452 pSW101; 3, free probe. Full-length (FL), read-through (RT), and S1-degraded probes (S1) are indicated. (B) DNA sequence of 155-bp *Nae*I fragment. The *Nae*I targets are underlined, the *amiE* and *amiB* open reading frames are translated, the stem of the T2 transcription terminator is shown by overlines, and the region of termination is shown by arrows.

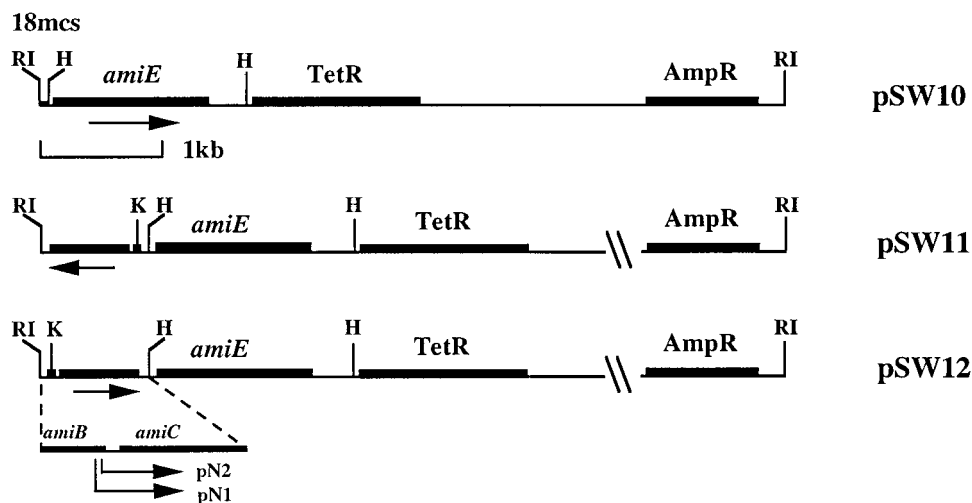


FIG. 5. Construction and use of promoter probe vector pSW10. The multiple cloning site (MCS) from M13mp18 was subcloned into *EcoRI-HindIII*-cut pBR322 to generate pSW9. The promoterless *amiE* gene from pAS20 was isolated on a *SmaI* fragment (Fig. 1A) and subcloned into the unique *HindIII* target of pSW9 by using *HindIII* linkers (pSW10). The 658-bp *XhoI* fragment from pAS20 (Fig. 1A) was subcloned into *SalI*-cut pSW10 to generate pSW11 and pSW12, which carry the insert in opposite orientations. The approximate locations of the *nr* promoters are shown in the exploded portion of pSW12, and the arrows indicate the direction of transcription from these promoters. Restriction sites are RI (*EcoRI*), K (*KpnI*), and H (*HindIII*); the drug resistance determinants of pBR322 are for tetracycline and ampicillin resistance, and the amidase structural gene (*amiE*) is identified.

bridized to the 1.3-kb *amiE*-specific transcript. This result suggests that under inducing conditions, transcription of *amiBCRS* results from read-through of a transcription terminator between *amiE* and *amiB*. The *amiEBCRS* transcript is approximately 5.0 kb in length, consistent with the identification of a potential transcription terminator immediately downstream of *amiS*.

S1 mapping downstream of the *amiE* gene. The results of the Northern analysis showed that transcription from pE gave rise to a 1.3-kb *amiE* transcript and an unstable 5.0-kb transcript, indicating partial termination of transcription downstream of *amiE*. The DNA sequence of the region between the end of the *amiE* gene and the start of the *amiB* gene has been determined and is shown in Fig. 4B. The sequence shows, between the two open reading frames, a potential transcription terminator sequence located between residues 1319 and 1343, with a GC-rich stem followed by a run of pyrimidine residues. To identify the transcription stop point(s) downstream from the *amiE* gene and to confirm that expression of *amiBCRS* arose from read-through of this terminator, S1 nuclease mapping was carried out. A continuously labelled single-stranded (*NaeI*, 156-base) probe, which extended over the region encompassing the inverted repeat sequence downstream of *amiE*, was used (Fig. 4B). In addition, the probe also contained 74 nucleotides of M13-derived sequence, arising from its preparation. The RNA used in these experiments was isolated from noninduced and lactamide-induced cultures of PAC452 pSW101, and the results of S1 mapping are shown in Fig. 4A.

The untreated full-length probe (FL, 230 bases) is shown in Fig. 3A, lane 3. Following S1 nuclease digestion of the RNA-DNA hybrids with the RNA from the noninduced cells, the probe is completely destroyed (lane 1). With the RNA from the induced culture (lane 2), a major band (RT, 156 bases), which corresponds to degradation of the M13 sequences but is otherwise completely protected, was seen. This shows that some transcripts read through the terminator downstream of *amiE*. No bands with sizes between those of the FL probe and the read through-protected fragment were seen, demonstrating that the S1 nuclease digestion went to completion. In ad-

dition, with the induced culture (lane 2), there were a series of bands (S1, 83 to 73 bases) which correspond to transcripts terminating at the T2 terminator (Fig. 4B). S1 nuclease mapping was also carried out using larger probes encompassing more downstream DNA, which were also shown to be fully protected to the same extent (results not shown). Scanning densitometry of the data in Fig. 4B indicated that approximately half of the transcripts are able to read through T2. These results together with the Northern analysis show that under inducing growth conditions the majority of *amiBCRS* transcripts originate from pE.

Analysis of promoter sequences upstream of *amiCR*. Previous studies with plasmids carrying *amiR* and increasing lengths of upstream sequence identified promoter activity between the *KpnI* (residue 2395) and *ClaI* (residue 3307) targets, some distance upstream of the gene itself (6) (Fig. 1A). Following the identification and sequencing of the *amiC* gene (residues 2528 to 3685), two potential RpoN-dependent promoter sequences were identified between the *KpnI* target and the 5' end of *amiC* (22). To further investigate the promoter activity in this region, a promoter probe vector was constructed using the *amiE* gene as a reporter.

The multiple cloning site from M13mp18 was subcloned into *EcoRI-HindIII*-cut pBR322 to create plasmid pSW9. The promoterless *amiE* gene was isolated from pAS20 on a 1.7-kb *SmaI* DNA fragment, *HindIII* linkers were added to the ends, and the fragment was subcloned into the unique *HindIII* site of pSW9, to generate the promoter probe vector pSW10 (Fig. 5). This vector is of general use in the analysis of promoter activity since the amidase assay is very sensitive, inexpensive, and easily performed with intact cells.

The 658-bp *XhoI* fragment from pAS20 which contains the 3' end of *amiB*, the two potential *rpoN*-dependent promoters, and the 5' end of the *amiC* gene was subcloned into the *SalI* target of pSW10. Derivatives of pSW10 were obtained with the fragment in both orientations (pSW11 and pSW12) (Fig. 5), and amidase activity was determined in *E. coli* carrying these plasmids. The promoter probe vector itself shows a background level of amidase expression (0.2 U), probably due to a

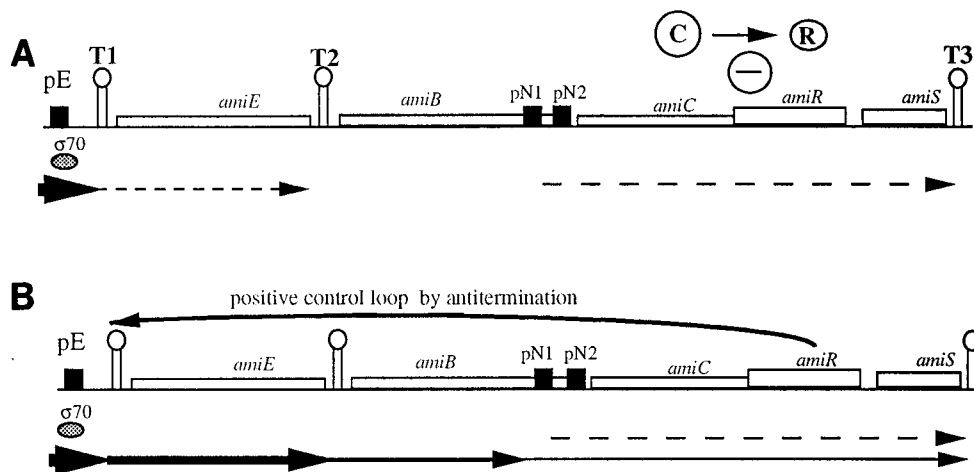


FIG. 6. Model of transcriptional regulation of the amidase operon of *P. aeruginosa*. The operon is shown in diagrammatic form, with arrow thickness representing the levels of transcription for growth under noninducing (A) and inducing (B) conditions. Promoter sequences are shown (■) and labelled. Transcription termination sequences are shown as stem loops and labelled, and the negative effect of AmiC on AmiR under noninducing conditions is shown diagrammatically. AmiR antitermination is RNA sequence dependent and occurs only at the T1 terminator.

low level of transcription from vector sequences. With pSW11 in which the 658-bp *XhoI* fragment is inserted into the vector in the direction opposite to that expected for promoter activity, no amidase activity is seen. With pSW12, in which the DNA fragment is placed in the opposite (correct) orientation, an average amidase activity of 0.5 U is seen. This is a low constitutive level of activity, which suggests that the DNA fragment carries a weak promoter firing in the correct orientation for expression of *amiCRS*.

DISCUSSION

A major tenet of the antitermination mechanism regulating expression of the amidase operon of *P. aeruginosa* is that transcription from the promoter should be at a strong constitutive level. We have now mapped the transcription start point, confirming the promoter identification, and shown that expression from the promoter is constitutive. The Northern analysis presented shows that under inducing conditions a major transcript of 1.3 kb, which encodes *amiE*, is produced. S1 nuclease mapping has shown that approximately half of the pE transcripts terminate at T2, a newly identified transcription terminator downstream of *amiE*, and the remainder continue through the rest of the operon, producing a polycistronic mRNA encoding *amiEBCRS*. The full-length transcript encoding all the amidase genes appears to be particularly unstable, which may ensure that excess regulatory and transport proteins are not produced when inducing amides are depleted.

During earlier subcloning and complementation studies with *P. aeruginosa*, it was shown that *amiR* could be expressed only from a vector promoter-independent site if a considerable length of upstream sequence was also present (6). These investigations clearly showed that *amiR* expression in *P. aeruginosa* was dependent on sequences present between *KpnI* (residue 2397) and *ClaI* (residue 3307) (Fig. 1A) (6). Subsequent DNA sequencing studies then identified two potential RpoN-dependent promoter sequences upstream of *amiC* (22). The construction and use of pSW10 as a promoter probe vector have now demonstrated that in *E. coli* the 658-bp *XhoI-XhoI* fragment contains a functional, albeit rather weak, promoter. We have attempted to map transcription start sites in this region, using primer extension and S1 mapping; however, the

analysis was confused by read-through transcription from pE, the results are not presented, and we are unable to draw any conclusions from the data obtained. However, the existence of a weak constitutive promoter upstream of *amiCRS* to provide sufficient amounts of AmiC and AmiR under noninducing conditions to trigger amidase expression on the addition of inducing amides. It is not clear from these results whether the observed promoter activity is due to pN1 and pN2 or other sequences on the 658-bp *XhoI* DNA fragment.

Initial studies of the amidase system showed that it was positively regulated by *amiR* (12), and subsequent investigations showed that this regulation occurred by an AmiR-mediated transcription antitermination mechanism (10). More recently, it has been shown that the transcription antitermination activity of AmiR is negatively regulated by AmiC via a protein-protein interaction (22). The results presented in this paper now allow us to propose a model to explain the transcriptional regulation of the amidase operon (Fig. 6). Under noninducing conditions, there will be constitutive transcription from the pE promoter, most probably regulated by catabolite repression, most of which is terminated at T1 with the remainder terminating at T2 (Fig. 6A). Thus, we expect that low levels of amidase activity are always present within the cells, as was observed for noninduced *P. aeruginosa* PAC452 pSW101 (22). In addition, there will be weak transcription from the promoter activity identified within the 658-bp *XhoI* fragment, upstream of *amiC*, which will provide low levels of AmiC, AmiR, and AmiS activity. Following induction (Fig. 6B), initially, AmiC inhibition of AmiR ceases, thus initiating a positive feedback loop whereby *amiR* increases its own expression and that of all the other genes in the operon via the antitermination reaction at T1. Under catabolite-derepressing inducing conditions, we would thus expect pE to function maximally, and with AmiR-mediated antitermination at T1, high levels of amidase will be produced. However, we would expect the T2 terminator to significantly reduce expression of *amiEBCRS* from pE. Under these inducing conditions, the weak promoter activity in the 658-bp *XhoI* fragment probably contributes very little to the overall levels of *amiCRS* expression. It is thus apparent that AmiR functions in a positive control loop and together with AmiC forms an amide-dependent autogenous control circuit.

In addition, since AmiR is involved in a positive feedback loop, with the native organization of the operon, amidase expression is subject to a high level of AmiR-dependent amplification under inducing conditions.

Despite the obvious clarity of the model, there are additional regulatory elements which remain uncharacterized. The first is the mechanism of the catabolite repression of amidase synthesis, since catabolite repression in *P. aeruginosa* is poorly understood at the molecular level. The second uncharacterized element is the finding that deletions around the unique *KpnI* target or removal of the 658-bp *XhoI* fragment leads to increased amidase activity. *Bal* 31-generated deletions from the unique *KpnI* target in pJB950 (*amiC*, constitutive: 4 U) were initially used to locate the *amiR* gene, and a series of deletion derivatives were obtained, all of which exhibited increased constitutive amidase activity (6). One plasmid, pDC11, with the smallest deletion of approximately 170 bp around the *KpnI* target, produced 16 U of amidase activity. Subsequently, a derivative of pJB950 with a deletion of the 658-bp *XhoI* fragment (pSW5) was made (22). The deletion in pSW5, which removes the 3' end of the *amiB* gene, including the pN1 and pN2 promoters and the 5' end of *amiC*, again leads to increased amidase activity of 8.7 U (22). These effects cannot be due to either the removal of the 5' end of *amiC*, since pJB950 is an *amiC* mutant, or the removal of *amiB* sequences, since we have shown that deletion mutations of *amiB* do not affect either amidase induction or the level of amidase expression (unpublished observations). In both of these cases we believe that the increased amidase activity can be due only to increased AmiR production and that the deletions must be removing a *cis*-acting transcriptional negative regulator, rather than just moving the *amiR* gene closer to the pE promoter.

A further complication of the model arises from investigations of pN1 and pN2 function. As a part of these studies, we have investigated amidase expression in both *E. coli* and *P. aeruginosa rpoN* mutants and found increased activities in both organisms (results not presented). Assays with *E. coli* ET8045 (*rpoN*) pJB950 showed activities similar to those with pDC11, in which pN1 and pN2 are deleted. At the moment, we are unsure whether the deletion results and the *rpoN* mutant results are observations of two aspects of the same regulatory system. However, it is tempting to suggest that the two phenomena are linked and that a single transcriptional negative regulatory system involving these promoter elements and RpoN is responsible for this effect.

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