Positive Control of *Pseudomonas aeruginosa* Amidase Synthesis Is Mediated by a Transcription Anti-termination Mechanism

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The DNA sequence of the region upstream from the amidase structural gene (*amiE*) of *Pseudomonas aeruginosa* indicates that amidase (EC 3.5.1.4) is transcribed from an *Escherichia coli*-like promoter located 150 bp before the *amiE* translation initiation codon. The sequence between the promoter and the coding sequence includes a single open reading frame followed by an *E. coli*-like rho-independent transcription terminator. A deletion within the presumed terminator region which disrupts the potential stem/loop formation leads to high constitutive amidase expression which is independent of the product of the regulator gene (*amiR*). It is proposed that the catabolic aliphatic amidase of *P. aeruginosa* is regulated by a transcription anti-termination mechanism. The magnoconstitutive mutant PAC433 has promoter and terminator sequences identical to the wild-type PAC1 but contains a single base pair change in the *amiE* gene ribosome-binding site.

INTRODUCTION

Expression of the amidase gene (*amiE*) of *Pseudomonas aeruginosa* is regulated by at least two independent control systems. Genetic and biochemical investigations have shown positive control by the product of the regulator gene (*amiR*) (Farin & Clarke, 1978; Drew, 1984) and genetic and physiological studies have shown the system to be subject to catabolite repression by succinate (Smyth & Clarke, 1975a). The amidase genes from the magnoconstitutive mutant PAC433 were cloned in bacteriophage λ (Drew *et al.*, 1980) and subcloned into plasmid pBR322 on a 5.3 kb DNA fragment, plasmid pJB950 (Clarke *et al.*, 1981). The *amiE* gene was found to lie close to one end of the DNA fragment and both the DNA and amino acid sequences of amidase have been determined (Brammar *et al.*, 1987; Ambler *et al.*, 1987). Deletion analysis and transcomplementation in *Escherichia coli* and *P. aeruginosa* have shown that the *amiR* gene is located in a 1.0 kb region about 2 kb downstream of *amiE* and transcribed in the same direction (Cousens *et al.*, 1987).

We have now sequenced the region upstream of *amiE* and measured amidase activity in a series of newly constructed deletion plasmids. This investigation has led to the proposal of a model for the positive regulation of amidase synthesis by a transcription anti-termination mechanism.

METHODS

Growth of bacterial strains. Table 1 lists the strains of *Escherichia coli* and *Pseudomonas aeruginosa* used in this study, and Table 2 lists the plasmids. *E. coli* strains were grown at 37 °C in L broth (Lennox, 1966) and *P. aeruginosa* strains were grown at 37 °C in Oxoid no. 2 broth. Antibiotics were used at the concentrations described previously (Cousens *et al.*, 1987). For amidase assays the media and growth conditions were as described previously (Drew, 1984).

Abbreviation: ORF, open reading frame.

Table 1. *Bacterial strains*

(a) <i>E. coli</i>			
Strain	Genotype (relevant features)	Reference	
JA221	<i>hsdR recA trp leu</i>	Clarke & Carbon (1978)	
JM103	$\Delta(lac-pro)$ <i>hsdR F' traD36 proAB lacI^a ZΔM15</i>	Messing <i>et al.</i> (1981)	

(b) <i>P. aeruginosa</i>			
Strain	Genotype	Amidase phenotype	Reference
PAC1	<i>amiR⁺ amiE⁺</i>	Inducible Ami ⁺	Kelly & Clark (1962)
PAC308	<i>amiR⁺ amiE8</i>	<i>AmiR⁺ AmiE⁻</i>	Brammar <i>et al.</i> (1967)
PAC433	<i>amiR1 amiE114,120</i>	Constitutive Ami ⁺	Smyth & Clark (1975 <i>b</i>)
PAC452	<i>ami-161</i>	AmiRE deletion	Day (1975)

Table 2. *Plasmids*

Plasmid	Size (kb)	Markers†	Reference
pJB950	9.1	Ap AmiRE	Clarke <i>et al.</i> (1981)
pCL43	6.1	Ap AmiE	
pCL50*	15.0	Sm AmiE	This study (see Fig. 1)
pCL51*	15.0	Sm AmiE	
pCL52	9.1	Ap AmiRE	
pCL53	9.1	Ap AmiRE	
pCL54	7.6	Ap AmiR	
pCL55	6.1	Ap AmiE	

* These plasmids are based on pKT231 (Bagdasarian *et al.*, 1981); other plasmids are based on pBR322 (Bolivar *et al.*, 1977).

† Phenotypic abbreviations are for resistance to ampicillin (Ap), streptomycin (Sm) and amidase structural (*amiE*) and regulator (*amiR*) genes.

DNA manipulations. Plasmid DNA preparations, restriction enzyme digestion, agarose gel electrophoresis, fragment recovery, ligations and transformations were as described previously (Drew, 1984; Cousens *et al.*, 1987).

Plasmid mobilization. Recombinant broad-host-range plasmids were constructed and characterized in *E. coli* JA221 and mobilized into *P. aeruginosa* strains using pTH10 as described previously (Drew, 1984).

Amidase assays. Amidase activity was measured in intact cells by the transferase assay (Brammar & Clarke, 1964) with acetamide as substrate. Activities presented are the mean values of duplicate assays carried out on at least three occasions. One unit (U) represents 1 μ mol acetohydroxamate formed min⁻¹.

DNA sequencing. DNA sequence was obtained from M13 mp8 and 9 recombinants by the chain termination reactions of Sanger *et al.* (1977) using ³⁵S-dATP (Biggin *et al.*, 1983). The PAC1 promoter fragment was obtained after HindIII/HindII digestion of pCL43, and the PAC433 fragment was sub-cloned from pDC5 (Cousens *et al.*, 1987). DNA cleavage of vectors, ligations, transfections, recombinant analysis, template preparations, sequencing reactions, polyacrylamide gel electrophoresis and autoradiography were essentially as described in the BRL M13 Cloning/Dideoxy Sequencing instruction manual. Free energy calculations for the termination loops were by the method of Freier *et al.* (1986) and the plot from the FOLD program of Zucker & Stiegler (1981).

RESULTS AND DISCUSSION

Investigation of promoter mutation

The organization and direction of transcription of the amidase genes in plasmid pJB950 are shown in Fig. 1 (*a*). Strain PAC433, from which the amidase genes were cloned, was described as a magnoconstitutive up-promoter mutant (Smyth & Clarke, 1975*b*). To investigate the promoter mutation two plasmids were constructed, pCL50 and pCL51 (Fig. 1 *b*), which carry the upstream sequences and *amiE* gene (*Hind*III-*Xho*I) from respectively the wild-type PAC1 and PAC433 cloned into the broad-host-range vector pKT231 (Bagdasarian *et al.*, 1981). The recombinant plasmids were identified and characterized in *E. coli* JA221. Neither of these constructs, which lack *amiR*, gave measurable levels of amidase activity in *E. coli*.

Fig. 1. Restriction map of *P. aeruginosa* DNA inserts in recombinant plasmids. Plasmids based on pBR322 are unlabelled, plasmids based on pKT231 are indicated *. Plasmids pCL50 and pCL43 carry DNA from the wild-type PAC1, pJB950 and pCL51 to pCL55 carry DNA from PAC433.

Table 3. Amidase assays of *P. aeruginosa* carrying plasmid pCL50 or pCL51

Strain	Phenotype†	Plasmid	Amidase activity*			
			Growth conditions:			
			Succinate	Succinate/ lactamide	Lactate	Lactate/ lactamide
PAC1	Ind Ami ⁺	—	0.1	8.1	0.1	2.5
PAC452	AmiRE deletion	—	0.1	0.1	0	0.1
PAC452		pCL50	0.8	0.8	0.8	0.8
PAC452		pCL51	4.8	5.2	4.3	4.3
PAC308	AmiR ⁺ , E ⁻	—	0	0	0	0
PAC308		pCL50	0.8	15.4	0.6	2.7
PAC308		pCL51	8.5	36.2	4.5	19.3

* Amidase activity [μmol acetohydroxamate formed min^{-1} (mg dry wt) $^{-1}$] was measured in intact cells by the transferase assay (Brammar & Clarke, 1964).

† Ind, inducible.

Plasmids pCL50 and pCL51 were mobilized into *P. aeruginosa* strains PAC452 (*amiER* deletion) and PAC308 (*amiE*⁻ *R*⁺) as described previously (Drew, 1984) and amidase activity was measured under inducing and non-inducing conditions (Table 3). In strain PAC452 amidase expression from pCL50 was at a low constitutive level [$0.8 \text{ U (mg dry wt)}^{-1}$], presumably due to the high copy number of the pKT231 vector. Expression from pCL51 in this strain was fivefold higher than from pCL50. In PAC308 amidase expression could be regulated from the chromosomal *amiR* gene. With pCL50 there was low expression in non-inducing growth conditions and high expression in the presence of inducer as expected. With pCL51 the non-induced level of activity was of the same order as that in PAC452 and in the presence of inducer very high amidase activity was found. Thus at least one of the mutations in strain PAC433 has been located to the *amiE* gene region and causes increased amidase expression in both the presence and absence of *amiR*.

Fig. 2. DNA sequence from strain PAC1 upstream of *amiE*. The sequence is written 5' to 3' and runs from the *Hind*III target to the *amiE* start codon (ATG: underlined). The *E. coli* consensus promoter sequence is shown 91-96 (-35) and 115-120 (-10). Translation of the ORF downstream of the promoter is shown. Arrows show the region of dyad symmetry comprising the transcription terminator, and the two boxed sequences are *Sma*I targets. Strain PAC433 carries a G for A substitution at position 249.

DNA sequence analysis of the amidase promoter region

The DNA sequences of the 315 bp *Hind*III-*Hind*II fragment from PAC1 and PAC433 were determined and part of this sequence is shown in Fig. 2. The DNA sequence runs from one end of the *P. aeruginosa* DNA fragment (*Hind*III) to the amidase N-terminal methionine and shows several interesting features. Some 150 bp upstream of the amidase translation start (262) is a consensus *E. coli* promoter sequence. This is followed by an open reading frame of 35 amino acids which stops at a rho-independent transcription terminator (Platt, 1981). The terminator sequence shows an axis of dyad symmetry which would produce a 16 bp GC-rich stem, with a four-residue loop followed by six T Residues (Fig. 3a). Downstream of the terminator sequence is a conventional ribosome-binding site followed by the amidase initiation codon. The only difference between the PAC1 and PAC433 sequences is a single base pair A→G change at position 249. This lies just upstream of *amiE* in the ribosome-binding region.

The interpretation of these findings is that RNA polymerase initiates transcription at a high frequency from the consensus promoter and that under non-inducing conditions the transcript terminates before *amiE*. Under inducing conditions *amiR*p would then allow transcription to proceed through the terminator into *amiE*.

Investigation of the transcription terminator

The *amiE* transcription terminator region contains two *Sma*I targets located 10 bp apart and pJB950 contains one additional *Sma*I target located downstream of *amiE* (Fig. 1a). Plasmid pJB950 was cut with the *Sma*I isoschizomer *Xma*I and religated. Plasmids pCL52, 53 and 54 were identified and characterized (Fig. 1c). Amidase activity was measured in *E. coli* JA221

Fig. 3. Predicted structures for the rho-independent terminators within the *amiE* leader. (a) Structure predicted for the wild-type terminator, pJB950; (b) structure predicted for the deleted terminator found in pCL53 and pCL55. Free energy (ΔG) calculations shown (Freier *et al.*, 1986) are expressed as kcal mol⁻¹ (1 kcal = 4.184 kJ).

carrying these plasmids under non-inducing conditions with glucose or succinate as carbon sources. The parental plasmid pJB950 gave low constitutive amidase expression [4.6 U (mg dry wt)⁻¹]; pCL51, which carries only the *amiE* gene, gave low background activity (0.1 U mg⁻¹), as did pCL52, which lacks the 10 bp *SmaI* fragment and has the 1.5 kb *amiE SmaI* fragment in reverse orientation (0.2 U mg⁻¹). This indicated that the DNA sequence just upstream of *amiE* did not carry the amidase promoter. Plasmid pCL53 is identical to pJB950 but lacks the 10 bp *SmaI* fragment in the terminator region. Amidase expression from pCL53 was at a high constitutive level (30.3 U mg⁻¹) approximately sevenfold higher than expression from pJB950. Thus disruption of the terminator region leads to elevated expression of amidase. For plasmid pCL54 it was considered possible that *amiR* would be expressed at high levels from the *amiE* promoter by readthrough transcription. This was tested by measuring amidase activity in *E. coli* JA221 carrying both pCL54 (*amiR* fusion) and pCL51 (*amiE*). The low level of amidase expression in this strain (0.4 U mg⁻¹) suggests the presence of termination sequences between the *SmaI* target (1700) and *amiR*. Plasmid pCL55 (Fig. 1c) was constructed by deletion of *amiR* and other downstream sequences from pCL53. This plasmid carries *amiE* in the normal orientation but the 10 bp *SmaI* fragment and the *amiR* gene are missing. Amidase activity from pCL55 was at a high constitutive level (27.9 U mg⁻¹) similar to that from pCL53. Thus the sequence changes to the transcription terminator have made expression independent of *amiR*.

Anti-termination model of amidase expression

The DNA sequence upstream of *amiE* shows the following features: (a) consensus *E. coli* promoter; (b) short open reading frame; (c) an *E. coli*-like rho-independent transcription terminator; (d) ribosome-binding site.

Results with pCL52 show that amidase is not expressed from a promoter located close to the gene itself, and changes which affect the structure of the terminator region (pCL55) cause

Fig. 4. DNA sequences of *bgl* and *amiE* leader regions upstream of terminator loops. (a) Sequence of the *bgl* leader region; the arrow shows the site of the 6 bp insertion; (b) sequence of the *amiE* leader region. Boxed sequences show homologous region.

amidase to be expressed at a very high level in the absence of *amiR*. The sequence upstream of *amiE* has the potential to code for an RNA hairpin loop structure characteristic of a rho-independent transcription terminator (Platt, 1981). The wild-type and PAC433 terminators have a calculated free energy of formation of $-23.3 \text{ kcal mol}^{-1}$ ($-97.5 \text{ kJ mol}^{-1}$) (Fig. 3a). In plasmids pCL53 and pCL55, both of which express amidase at high levels, the termination loop has a much reduced free energy of formation of $-9.7 \text{ kcal mol}^{-1}$ ($-40.6 \text{ kJ mol}^{-1}$) (Fig. 3b) (Freier *et al.*, 1986). Strain PAC433 was originally considered to be an amidase constitutive up-promoter mutant with reduced sensitivity to catabolite repression by succinate. Cotransduction studies showed that the mutation conferring resistance to catabolite repression was closely linked to *amiE* (Smyth & Clarke, 1975b). One of the mutations in PAC433 has now been located to the *amiE* gene ribosome-binding site and shown to cause a fivefold increase in amidase expression. PAC433 shows high amidase activity under all growth conditions [about $40 \text{ U (mg dry wt)}^{-1}$] (Cousens *et al.*, 1987). Expression from the cloned PAC433 genes in the multicopy plasmid pJB950 in *E. coli* is at a much reduced level (about 4 U mg^{-1}). The low activity in *E. coli* is thought to be due to very poor *amiR* expression, since provision of excess *amiR* in *trans* leads to high amidase activity (about 60 U mg^{-1}) (Cousens *et al.*, 1987). This compares with the 30 U mg^{-1} found from pCL55 in *E. coli*.

The mechanism of action of *amiRp* in antitermination has not yet been characterized. However, the best-characterized model of anti-termination occurs in coliphages, where, mechanistically, it appears that an anti-termination protein interacts with RNA polymerase at utilization sites located between promoter and transcription terminator (Friedman & Gottesman, 1983). Within the amidase leader region there are no sequence homologies with published *nut* and *qut* sequences. However, Mahadevan & Wright (1987) have shown that regulation of the *bgl* operon of *E. coli* occurs by transcription anti-termination. In this system there are no apparent coliphage utilization sites, but these authors have shown that a six base pair insertion 12 nucleotides upstream of the termination loop renders the system uninducible. This presumably defines at least one site involved in the anti-termination process. The corresponding region of the *amiE* leader sequence shows some homology to the *bgl* leader region (Fig. 4). The homology is structural to the left of the insertion site and direct to the right. It is possible that the shared motifs represent some essential features for the anti-termination process. At present there is no direct evidence that this part of the *amiE* leader region is involved in the anti-termination process; however, the results presented show that the positive control of amidase synthesis occurs by a transcription anti-termination mechanism mediated by *amiRp*. This mechanism is being further investigated by studies of the wild-type inducible system.

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