REGULATION OF *Pseudomonas aeruginosa* AMIDASE EXPRESSION BY *amiC*

by

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Abstract

Regulation of amidase synthesis in *P.aeruginosa* has been studied using cloned genes from strains PAC1 and PAC433. Using *in vitro* constructed rearrangements, deletions and insertions, a new gene *amiC* has been identified between the amidase structural gene (*amiE*) and the positive regulator gene (*amiR*). Transcomplementation studies in *E.coli* and *P.aeruginosa* and analysis of *in vitro* constructed *amiC*$^-$ mutants has shown that AmiC is a negative regulator of amidase expression. The DNA sequence of the *amiC* region has been determined and two potential *ntrA* dependent promoters identified upstream of *amiC.*

amiC and *amiR* expression vectors were constructed for complementation analysis in *E.coli* and *P.aeruginosa.* Using the *amiC* expression vector in *P.aeruginosa*, AmiC was purified to homogeneity. The purified AmiC was shown to have protein kinase activity *in vitro.* A model has been proposed whereby the transcription antitermination activity of AmiR is modified by AmiC dependent phosphorylation.

DNA sequencing studies completed the sequence of the entire 5.3kb *Hin*dIII-*Sal*I *P.aeruginosa* DNA fragment containing the amidase genes and two additional open reading frames were identified, giving the gene order for the operon, *amiEYCRX.* A transcription terminator was identified downstream of *amiE.* Transcript analysis of the amidase operon has shown constitutive expression from the *amiE* σ^{70} - dependent promoter. Under inducing conditions, appproximately 40% of the *amiE* transcripts read through the downstream terminator sequence and into *amiY, C, R, X.*

Studies designed to investigate the role of the *ntrA* dependent promoters failed to identify transcripts starting from these positions. However amidase expression from the cloned genes was shown to be regulated by the enteric *ntr* system. These investigations showed that σ^{54}-holoenzyme functions to down-regulate amidase expression in *E.coli* and *P.aeruginosa.* The roles of AmiY and AmiX have not been established. However AmiY contains a consensus nucleotide binding domain and amiX shows the characteristics of an integral membrane protein.

Acknowledgments

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ABBREVIATIONS

ATP	adenosine triphosphate
BRL	Bethesda Research Laboratories
BSA	bovine serum albumin
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
IPTG	isopropyl-β-D-thiogalactopyranoside
MOPS	3-[N-morpholino] propanesulphonic acid
NTP	nucleoside 5'-triphosphate
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethylsulfonylfluoride
RBS	ribosome binding site
RNA	ribonucleic acid
RNAase	ribonuclease A
SDS	sodium dodecyl sulphate
SSC	sodium saline citrate
TE	Tris-EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris(hydroxymethyl)-aminomethane
UV	ultraviolet

Throughout this work the names of genes have been written in italics for example, *amiC* , and the protein product of the gene has been written in a normal typeface with a capital letter at the begining for example, AmiC.

CHAPTER ONE - INTRODUCTION

1.1 *Pseudomonas* biology

The genus *Pseudomonas* comprises a large number of bacterial species of scientific, medical and industrial importance. Their habitats include soil, freshwater and marine environments. Some species are plant pathogens and one strain, *Pseudomonas aeruginosa* is an important human pathogen, causing lethal infections in the lungs of cystic fibrosis patients (Govan and Harrison, 1986). As an opportunistic pathogen, *P.aeruginosa* can also be responsible for eye infections, ear infections and is particularly associated with skin burns.

Pseudomonas strains are characterised as follows. They are Gram-negative, aerobic, non-spore forming, polarly flagellated, highly motile bacteria, except for one species (*P. mallei*), which is permanently nonflagellated. One hallmark of the Pseudomonads is their great nutritional versatility and they are among the most active participants in the process of mineralisation of organic matter in nature (Palleroni, 1975). The organic compounds used by many *Pseudomonas* species, to provide both carbon and energy sources, include hydrocarbons, carbohydrates, aliphatic acids, amines, amides, amino acids, aromatic compounds and alcohols. The utilisation of both natural and synthetic organic compounds by Pseudomonads has been investigated extensively, since industrially useful compounds can be synthesised via complex metabolic pathways from simple, frequently cheap carbon sources. The genes required for many of these complex catabolic biotransformations are plasmid encoded. For example, the plasmid NAH7 , which encodes the metabolism of napthalene (Schell, 1990) and plasmid TOL pWWO, encoding toluene degradation (Burlage *et al,* 1989).

The chromosomal organisation of genes in *Pseudomonas* species has been extensively studied by Bruce Holloway and circular chromosomal maps have been constructed for both *P. aeruginosa* and *P. putida* by using time-of-entry data combined with transduction analysis. These studies used chromosome-mobilising

plasmids (FP2, FP5 and R68.45) and the transducing phage F116L and G101. The result is a chromosomal map with over 300 markers for *P.aeruginosa* (Holloway & Zhang, 1990).

More recently, physical maps of the chromosomes of these two organisms have been constructed by analysis of restriction enzyme fragments by pulsed field gel electrophoresis. In *P.aeruginosa* PAO, 37 *Spe*I fragments have been identified ranging in size from 10 to 525 kilobases (kb), giving a total genome size of 5862 kb. For *P. putida*, 27 *Spe*I fragments have been identified, giving a total genome size of 5620 kb. A large number of genes have now been mapped to specific restriction fragments, and this information has allowed accurate comparisons of the chromosomal organisation in these two *Pseudomonas* species (Ratnaningsih *et al*, 1990).

The chromosomal organisation of *Pseudomonas* species shows substantial differences in the organisation of the genes compared with the *Enterobacteriaceae* (Bachmann, 1987). In particular, for *P.aeruginosa*, genes determining the pathways for the synthesis of amino acids, purines and pyrimidines are unlinked unlike the major operons seen in the enterobacteria. The classic example of this phenomenon are the *trp* genes encoding the enzymes for tryptophan biosynthesis. In *E.coli* the *trp* genes form a coordinately regulated single operon controlled by repression-attenuation. In *P.aeruginosa* the genes are found in four different chromosomal locations and show three distinctly different regulatory mechanisms (Crawford, 1986).

1.2 Gene cloning in *Pseudomonas*

1.2.1 Introduction

The discovery of type II restriction enzymes has allowed the *in vitro* manipulation of DNA sequences and this has proved one of the most powerful tools for investigations of gene regulation in bacteria. DNA manipulations are reliant on a suitable host for propagation of recombinant DNA molecules and cloning vectors to carry DNA fragments of interest. Host-vector systems have been most extensively developed for *E.coli* and consequently these have been most widely used for genetic manipulations.

However, there are problems associated with host-vector systems based on *E.coli.* (i) The transcription-translation machinery of *E.coli* recognises the transcription and translation signals of some other bacteria poorly. Consequently heterologous genes placed in an *E.coli* host are sometimes poorly expressed unless the appropriate *E.coli* - specific signals are provided. ii) Studies of heterologous operons may be difficult in *E.coli* because of differences in the respective global control networks, for example, catabolite repression or because of the absence of ancillary activator genes required for the operon expression iii) Foreign protein products produced in *E.coli* can be toxic to the cell, resulting in instability of the cloned DNA (Morales *et al,* 1990).

1.2.2 Host strains for gene cloning in *Pseudomonas*

An appropriate host strain must fulfil the following requirements;- i) can be rendered "competent" for transformation, allowing the efficient uptake of recombinant DNA molecules; ii) is restriction deficient, thus preventing degradation of foreign DNA; iii) is defective in general recombination functions, ensuring the integrity of recombinant plasmids is maintained; iv) is free of endogenous plasmids which could confuse the analysis of newly introduced plasmids.

Strains of *P. aeruginosa* and *P. putida* are now available which fulfil some of the requirements set out above. Bagdasarian and Timmis (1982) have developed transformation protocols for *P.aeruginosa* strain PAO1162 and *P. putida* KT2440, with plasmid RSF1010 DNA routinely yielding $>10^5$ transformants/ μg of DNA. Restriction and modification (*rmo)* defective strains of *P.aeruginosa* and *P. putida* have been isolated (Bagdasarian *et al,* 1981). In addition, the *recA* gene of *P.aeruginosa* has now been cloned and sequenced, and *recA*$^-$ strains of *P.aeruginosa* have been constructed by transposon mutagenesis (Ohman *et al,* 1985; Sano and Kageyama, 1987).

1.2.3 Cloning vectors for *Pseudomonas*

The construction of cloning vectors for *Pseudomonas* has concentrated on two replicons, RK2 and RSF1010. RK2 is a large, low copy number, self transferable plasmid which required extensive manipulations to make it "user friendly".

Unfortunately, these manipulations resulted in the loss of some functions required for stability and maintenance of the plasmid. Thus RK2 derived vectors are unstable and not generally used (Schmidhauser and Helinksi, 1985).

The broad host range, IncQ plasmid RSF1010 is relatively small (8684bp) (Scholz *et al,* 1989), carries genes for streptomycin and sulfonamide resistance and has an intermediate copy number of 13 copies per cell (Frey and Bagdasarian, 1989). Since the plasmid has a broad host range this means it can be used for genetic reconstructions in *E.coli* and then mobilised into *Pseudomonas* hosts. The plasmid is stably maintained in both *E.coli* and *Pseudomonas* and has been extensively manipulated to provide a wide range of cloning vectors for use in *Pseudomonas* , including cosmids, promoter probe vectors and expression vectors (Morales *et al,* 1990). Table 1.1 shows some of the broad host range cloning vectors that have been constructed from IncQ group plasmids (predominantly RSF1010) for gene cloning in *Pseudomonas.*

1.3 The control of gene expression in bacteria

Bacteria have evolved elegant control mechanisms for the production of specific proteins in response to inducer molecules and environmental signals. There are several points at which the production of a given protein can be regulated, dictated by the mechanism of protein synthesis. The first and most widely used mechanism, is to control the level of mRNA production, or transcription (see section 1.3.1). A second mechanism is to control the stability of the mRNA, once it is produced (King and Schlessinger, 1987). Thirdly, the rate of translation from mRNA to protein can be regulated (McCarthy and Gualerzi, 1990) and finally the stability of the final protein product can be regulated (Gottesman, 1987). These control systems are not mutually exclusive and frequently a combination of mechanisms is encountered. Since the control of mRNA production appears to be the most widely used mechanism for the regulated production of proteins, this will be considered in more detail.

Table 1.1. Properties of IncQ-derived broad host range vectors

Vector	Size (kilobase pairs)	Markers	Cloning site(s)	Insertional change
General-type cloning				
pKT210	11.8	Cm, Sm	*Sst*I	$Sm^r \rightarrow Sm^s$
		mob$^+$	*Eco*RI	$Sm^r \rightarrow Sm^s$
			*Hin*dIII	None
pKT231	13.0	Sm, Km	*Sst*I, *Sst*II	$Sm^r \rightarrow Sm^s$
		mob$^+$	*Eco*RI, *Hpa*I	$Sm^r \rightarrow Sm^s$
			*Hin*dIII, *Xho*I	$Km^r \rightarrow Km^s$
			*Cla*I, *Xma*I, *Pvu*I	$Km^r \rightarrow Km^s$
			*Bam*HI, *Bgl*II	None
pKT248	12.4	Cm, Sm	*Eco*RI	$Sm^r \rightarrow Sm^s$
		mob$^+$	*Sst*I, *Sst*II	$Sm^r \rightarrow Sm^s$
			*Sal*I	$Cm^r \rightarrow Cm^s$
pDSK509	9.1	Km, Sm	*Pst*I, *Sal*I, *Xba*I	$Sm^r \rightarrow Sm^s$
		mob$^+$	*Bam*HI, *Kpn*I	$Sm^r \rightarrow Sm^s$
			*Sst*I, *Eco*RI	$Sm^r \rightarrow Sm^s$
Joint replicon				
pKT230	11.9	Km, Sm	*Bam*HI	None
		mob$^+$	*Eco*RI, *Sst*I, *Sst*II	$Sm^r \rightarrow Sm^s$
		pACYC177	*Hin*dIII, *Sma*I, *Xho*I	$Km^r \rightarrow Km^s$
pFG7	13.3	Ap, Sm	*Sst*I, *Sst*II	$Sm^r \rightarrow Sm^s$
		Su, Tc	*Sst*I, *Sst*II	$Sm^r \rightarrow Sm^s$
		mob$^+$	*Bam*HI, *Cla*I	$Tc^r \rightarrow Tc^s$
		pBR322	*Hin*dIII, *Sal*I	$Tc^r \rightarrow Tc^s$
pUI81	13.3	Sm, Su, Tc	*Sst*I, *Sst*II	$Sm^r \rightarrow Sm^s$
		mob$^+$	*Bam*HI, *Cla*I	$Tc^r \rightarrow Tc^s$
		pBR322	*Hin*dIII, *Sal*I	$Tc^r \rightarrow Tc^s$
pMW79	13.3	Ap, Sm, Tc	*Sst*I	$Sm^r \rightarrow Sm^s$
		mob$^+$	*Sst*II	$Sm^r \rightarrow Sm^s$
		pBR322	*Bam*HI, *Cla*I	$Tc^r \rightarrow Tc^s$
			*Hin*dIII, *Sal*I	$Tc^r \rightarrow Tc^s$
pSUP104	9.5	Cm, Tc	*Eco*RI	$Cm^r \rightarrow Cm^s$
		mob$^+$	*Bam*HI, *Hin*dIII, *Sal*I	$Tc^r \rightarrow Tc^s$
		pACYC184	*Pst*I	None
Cosmid				
pMMB33, pMMB34	13.8	Km, *cos*	*Bam*HI, *Eco*RI	None
		mob$^+$	*Hpa*I, *Sst*I, *Sst*II	None
pFG6	15.3	Ap, Tc	*Bam*HI, *Cla*I	$Tc^r \rightarrow Tc^s$
		mob$^+$, *cos*	*Hin*dIII, *Sal*I	$Tc^r \rightarrow Tc^s$
pSUP106	9.9	Cm, Tc	*Eco*RI	$Cm^r \rightarrow Cm^s$
		mob$^+$, *cos*	*Bam*HI, *Hin*dIII	$Tc^r \rightarrow Tc^s$
		pACYC184	*Sal*I	$Tc^r \rightarrow Cm^s$
pJRD215	10.2	Km, Sm	*Bam*HI, *Kpn*I, *Mlu*I, *Hpa*I, *Nde*I, *Stu*I, *Xba*I, *Spe*I, *Eco*RI	None

Table 1.1. - *continued.*

Vector	Size (kilobase pairs)	Markers	Cloning site(s)	Insertional change
Promoter probe				
pKT240	12.9	Km, Ap *mob*$^+$	*Eco*RI, *Hpa*I	Sm^s → Sm^r
pCF32	15.1	Km, Ap *mob*$^+$	*Hin*dIII	*xylE* → *xylE*$^+$
pUI523A, pUI533A	9.7	Tc, *mob*$^+$, *lacZ*	*Kpn*I, *Dra*I, *Stu*I, *Sma*I	Fusion to *lacZ*
Controlled expression				
pMMB66EH	8.8	Ap, P_{tac}	*Eco*RI, *Sma*I, *Bam*HI	None
		mob$^+$, *lacI*^{q}	*Sal*I, *Pst*I, *Hin*dIII	None
pMMB67EH	8.8	Ap, P_{tac}, *mob*$^+$	*Eco*RI, *Sst*I, *Kpn*I, *Sma*I, *Bam*HI, *Xba*I	None
		lacI^q	*Sal*I, *Pst*I, *Sph*I, *Hin*dIII	None
pMMB207	9.0	Cm, P_{tac}, *mob*$^+$	Same as pMMB67EH	None
pMMB206	9.3	Cm, *mob*$^+$, *lacI*^{q}, P_{tac}-P_{lac}, *lacZ*α	Same as pMMB67EH	*lacZ*α$^+$ → *lacZ*α
pNM185	14.0	Km, *mob*$^+$, Pm, *xylS*	*Eco*RI, *Sst*I, *Sst*II	None
pERD21	13.8	Km, *mob*$^+$	*Eco*RI, *Sst*I, *Sst*II	None
		Pm, *xylS*	*Kpn*I, *Sal*I, *Hpa*I, *Bcl*I	None
pPLGN1		Km, P_L *mob*$^+$, *c*I857	*Eco*RI	None

Taken from Morales *et al,* 1990.

1.3.1 The general principles of positive and negative regulation

The general theory of gene expression proposed by Jacob and Monod (1961) was one of negative control and suggested that the structural genes for catabolic enzymes were controlled by regulator genes which produced repressor proteins. Such repressor proteins were thought to bind to regions of DNA known as operators, thus blocking the interaction of RNA polymerase with promoter sequences by steric hindrance. By this model, inducing substrates were proposed to bind to the repressor, causing a change in protein structure such that their interaction with operator sites was prevented. In the absence of repressor bound to the operator, RNA polymerase was then able to interact with the promoter sequences, initiating transcription.

The theory of repressors and operators proposed by Jacob and Monod (1961) rapidly achieved the status of a universal paradigm for gene expression. It was not until the work of Ellis Englesberg and his co-workers on the genes determining arabinose catabolism in *E.coli* that a true challenge to the universality of repressor-operator control appeared. The genetic data this group of workers accumulated led to the proposal in 1963, that the product of the regulator gene, *araC*, was a positive control factor, rather than a repressor (Helling & Weinberg, 1963).

With positive control the structural gene(s) requires the presence of the inducing molecule and the functional regulator gene for expression. The regulator gene in this situation would stimulate transcription from a promoter by RNA polymerase, generally by increasing the rate, or extent of open complex formation (Raibaud & Schwartz, 1984). In a simple situation the activity of the regulator gene would be determined by the presence or absence of an inducer molecule. There are many variations on the theme of positive and negative control and the two systems are not mutually exclusive, indeed many structural genes are regulated by a combination of positive and negative control. The archetypal system which demonstrates both positive and negative control is the *lac* operon of *E.coli*.

1.3.2 The *lac* operon of *E.coli* - An example of positive and negative control

The *lac* operon of *E.coli*, determining the metabolism of lactose has played a fundamental role in the development of molecular biology. In the late 1950s and early 1960s, studies with the *lac* system led to an understanding of genetic regulation, to the concept of mRNA production, and to the model of repressor-operator control.

The operon comprises a regulatory gene, *lacI*, which encodes a repressor which functions as a tetramer of 152kDa (Miller, 1978) and controls the coordinate expression of three structural genes, *lacZ, lacY* and *lacA* (Beckwith & Zipser, 1970). The organisation of the genes is illustrated in Figure 1.1. The structural genes are transcribed into a single polycistronic mRNA from a promoter just upstream of *lacZ. lacZ* codes for the enzyme β-galactosidase, whose active form is a tetramer of 500 kDa. *lacY* codes for a lactose permease with a molecular weight of 46.5 kDa (Buchel *et al*, 1980) and is an integral protein of the cytoplasmic membrane (Fox & Kennedy, 1965). *lacA* codes for a thiogalactoside transacetylase which is a dimer composed of identical subunits of molecular weight 22.7 kDa (Fowler *et al*, 1985). The physiological function of this protein remains unclear.

The expression of the *lac* operon is subject to both positive and negative control. *lacI* is expressed from its own promoter at a low constitutive level. The negative control system works as follows. In the absence of an inducer, such as lactose or certain other galactosides, the *lac* repressor is bound to the *lac* operator, preventing access and binding of RNA polymerase to the *lac* promoter. When an inducer binds to the repressor, its conformation is allosterically altered, and this prevents it from binding to the *lac* operator. Thus RNA polymerase interacts with the *lac* promoter and initiates transcription of the structural genes (Beckwith, 1987).

When an inducer such as lactose is used, there are several steps which are necessary for induction to take place. Firstly, lactose must be taken up by the cell

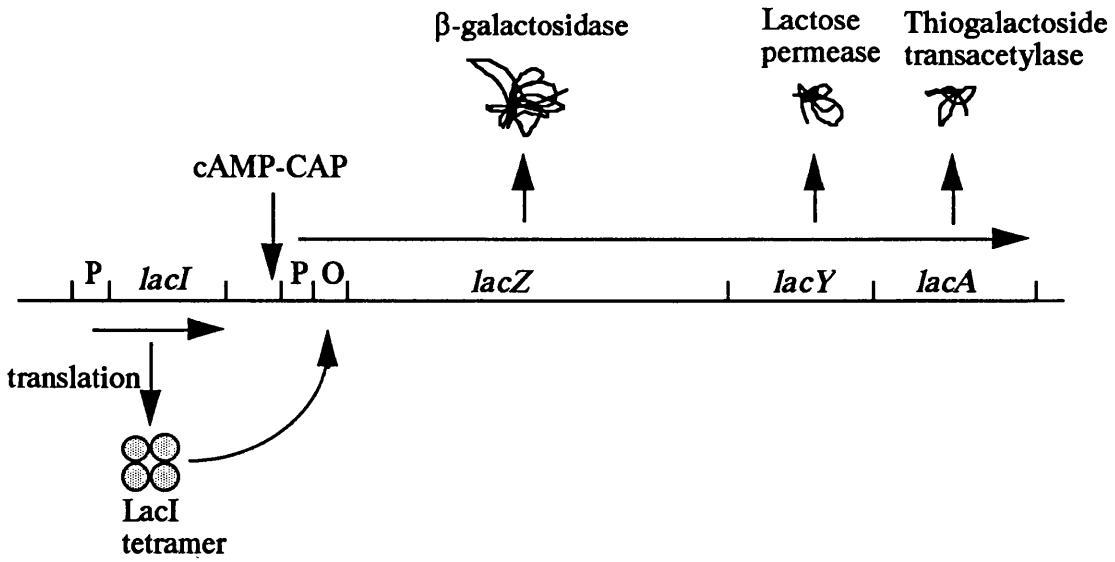


Figure 1.1. Organisation of the *lac* operon of *E.coli.* P= promoter, O= operator. The LacI repressor binds to the *lac* operator in the absence of inducer, preventing transcription of *lacZYA*. In the presence of inducer LacI does not bind to the operator thus allowing *lacZYA* transcription to proceed. cAMP-CAP binding stimulates transcription from the *lacZYA* promoter.

by the lac permease. Secondly, lactose must be converted to the true inducer (allo-lactose) by the transgalactosidation activity of β-galactosidase (Beckwith, 1987). Consequently the question is raised as to how the system is ever induced, since the negative control system described above suggests that no *lacZYA* expression occurs under non-inducing conditions. This is not in fact the case. The *lac* repressor is not infinitely tightly bound to the operator site on the DNA, and occasionally the operator is free. Thus a basal constitutive level of transcription of *lacZYA* occurs, which provides enough lac permease and β-galactosidase for induction of the operon (Kennell and Riezman, 1977).

Monod (1947) first noted that the presence of glucose in the growth medium, prevented the induction of the *lac* operon by lactose. There are two reasons for this phenomenon. Firstly, growth on glucose causes inducer exclusion by activation of an inhibitory system for the *lacY* transport system and other transport systems (Osumi & Saier, Jr., 1982). Secondly, growth on glucose reduces the level of cyclic AMP (cAMP) in the cells. This latter phenomenon is termed catabolite repression (Magasanik, 1961) and represents a global control network found in many bacterial species, affecting the expression of a variety of catabolic pathway genes

Catabolite repression of the *lac* operon functions as a positive control system. cAMP acts to promote transcription from the *lac* promoter by binding to the CAP (catabolite gene activator protein) protein and causing an allosteric transformation (Krakow & Pastan, 1973). In its altered conformation CAP binds to a site upstream of the RNA polymerase binding site (Beckwith *et al*, 1972; Majors, 1975) (see Figure 1.1). In the absence of CAP there is a weak affinity between RNA polymerase and the *lac* promoter (Hopkins, 1974), allowing a basal level of transcription of approximately 2% of the maximal level (Beckwith *et al*, 1972). In its activated state, CAP stimulates the binding of RNA polymerase to the *lac* promoter causing a large increase in transcription. In this way both positive and negative control mechanisms are used together to control the expression of the *lac* operon. In the following section, an account of the types of promoters used in *P. aeruginosa* is described and the ways in which they are controlled.

1.3.3 Bacterial promoters - emergent classes in *Pseudomonas* and other bacteria

A promoter is defined as the recognition sequence in DNA for the RNA polymerase-sigma factor complex. Following RNA polymerase binding, closed to open complex formation occurs, leading to the initiation of transcription. The specificity of RNA polymerase holoenzyme for a given promoter sequence is determined by the type of sigma factor bound. In *E.coli*, the most commonly used sigma factor is σ^{70}, which recognises the following consensus sequence (Helmann and Chamberlin, 1988):

$$\underset{-35}{\text{TTGACA}}\text{-}N_{17\pm1}\text{-}\underset{-10}{\text{TATAAT}}$$

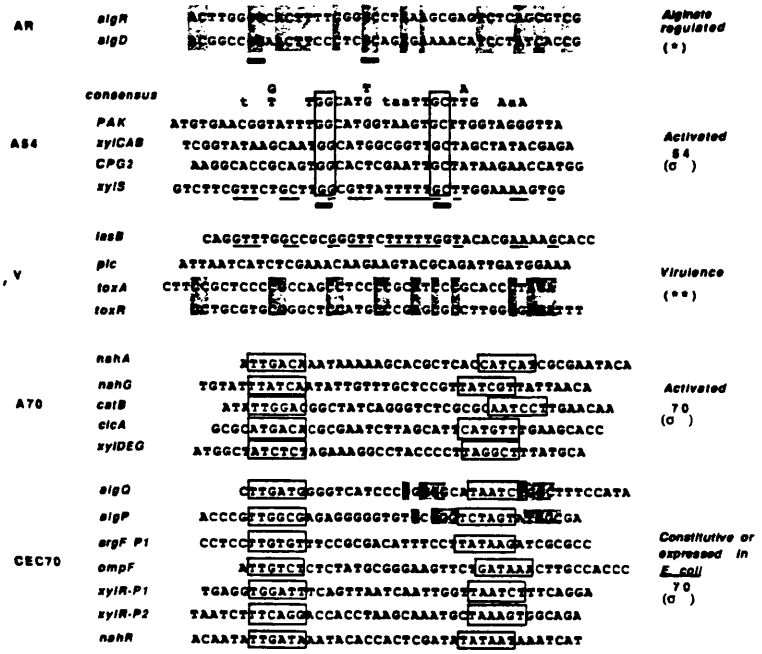
There are well characterised examples of alternative sigma factors, directing expression of particular sets of genes, for example σ^{29}, σ^{30} which are involved in the sporulation process in *Bacillus subtilis* (Helmann & Chamberlin, 1988) and σ^{32} in *E.coli* which directs the transcription of heat-shock genes from specific heat shock promoter elements (Cowing *et al*, 1985).

A large number of *Pseudomonas* promoters have now been identified and it has become clear that as with other bacteria, there are classes of promoters which are recognised by different sigma factors (Deretic *et al*, 1989). Table 1.2 shows a compilation of *Pseudomonas* promoters and their subdivisions into various classes.

The largest group of promoters identified in *Pseudomonas* are related to the σ^{70} dependent *E.coli* consensus promoter. This group of promoters can be further subdivided into two distinct classes:

i) the CEC70 class, represent promoters with sequences closely resembling the consensus σ^{70} promoter sequences of *E.coli*. These promoters are generally well recognised in *E.coli* and transcription from them is constitutive. The *amiE* promoter which is identified in this thesis falls into this class of promoter. There are two exceptions which do not fall strictly into this class; *argF*, which is expressed in *E.coli* (Itoh *et al*, 1988) but might have regulated expression in *Pseudomonas* and *NahR*, a LysR family member, by analogy with other members of this class

Table 1.2. A compilation of *Pseudomonas* promoters.



The DNA sequences shown are from +1 (mRNA start) to -40 (upstream). Promoters are organised into functional groups designated AR, A54, A70 and CEC70. The names of the groups reflect the type of regulation and proposed *P.aeruginosa* σ factors used, as indicated on the right hand side of the Table. Canonical sequences recognised by σ^{54} (GG-10bp-GC) or σ^{70} (TTGACA-17±1-TA-TAAT) are boxed. A tentative consensus for the A54 group of promoters in *Pseudomonas* is shown. Shaded areas indicate homologous sequences within a group of promoters. * and ** indicate sequences which either strongly depart from the σ^{54} or σ^{70} consensus sequences, or might be transcribed by unknown σ factors. Thick lines underscore GG-10bp-GC sequences in the *algD* and *algR* promoters which resemble σ^{54} sequences but are shifted further upstream from the usual -24 and -12 positions. Thin underlining exemplifies homologies of the *lasB* promoter with certain mem-bers of the A54 class. Taken from Deretic *et al* (1989).

(Chang *et al*, 1989), which may be negatively autoregulated.

ii) The second class (A70) have some similarity to the consensus σ^{70} promoter of *E.coli* and are thought to use the σ^{70} homologue in *Pseudomonas.* Transcription from this type of promoter appears to be positively regulated by genes (*nahA, nahG, catB, clcA)* which have homology with the LysR family of bacterial activator proteins (Henikoff *et al*, 1988). Promoters which fall into the A70 class do not appear to have the well conserved spacing and sequences around the -10 and -35 regions associated with the consensus σ^{70} sequence of *E.coli* which might explain the requirement for activators (Deretic *et al*, 1989).

A third, distinct class of promoters are the *algD-algR* group (AR) and the promoters controlling several other virulence determinants in *P.aeruginosa.* Within these classes there is considerable homology between promoter sequences and yet despite some homology with σ^{54} dependent promoter sequences (see below), the critical nucleotides at -12 and -24 recognised by σ^{54} are either absent or wrongly positioned with respect to the start of transcription. This may indicate an alternative type of sigma factor recognises these virulence promoters in *P.aeruginosa* (Deretic *et al*, 1989). Recently it has been shown that transcription from the *algR* and *algD* promoters is unaffected in a *ntrA*$^{-}$ *Pseudomonas* host, confirming that this sigma factor is not involved in transcription from these promoters (Mohr *et al*, 1990).

The most distinctive class shown in Table 1.2 are a set of activatable promoters (A54) proposed to be transcribed by the *Pseudomonas ntrA*(σ^{54})-like RNA polymerase holoenzyme. This is based on the presence of sequences similar to promoters known to be recognised by this sigma factor in *E.coli* and *Klebsiella spp.* Transcription from this type of promoter is regulated by a novel mechanism and this will be described in more detail below.

1.3.4 σ^{54} promoters and sensory pairs of proteins

a) Introduction

In recent years, a new type of bacterial sigma factor has been identified, called σ^{54}, encoded by the gene *ntrA* (*glnF, rpoN*). This sigma factor was originally identified as a positive regulatory factor required for the expression of glutamine synthetase in enteric bacteria (Garcia *et al,* 1977). Subsequent studies showed it to be required for the expression of other genes involved in nitrogen assimilation and nitrogen fixation, including the *nif* genes from *Klebsiella pneumoniae* (Dixon, 1984; Gussin *et al,* 1986) and *Rhodobacter capsulatus* (Jones and Haselkorn, 1989).

More recently it has become clear that in *Pseudomonas* and other bacteria, σ^{54} - holoenzyme is involved in the transcription of genes with diverse metabolic functions. For example an *ntrA*$^-$ genotype in *Pseudomonas putida* affects the utilisation of nitrate, urea and uncharged amino acids as nitrogen sources and C_4-dicarboxylates and α-ketoglutarate as carbon sources for growth (Kohler *et al,* 1989). Other examples of σ^{54} dependent genes include: i) the *fdhF* gene of *E.coli*, which encodes anaerobically inducible formate hydrogen lyase (Birkmann *et al,* 1987), ii) genes on the TOL plasmid of *Pseudomonas putida*, involved in the degradation of toluene and xylenes (Dixon, 1986; Ramos *et al,* 1987), iii) the pilin gene of *P.aeruginosa* (Ishimoto & Lory, 1989), iv) the *dctA* gene of rhizobia, which encodes a transport component for dicarboxylic acids (Ronson *et al,* 1987a).

The characteristic promoter sequences recognised by σ^{54} were first determined by analysis of the *nif* gene promoters of *Klebsiella pneumoniae* (Beynon *et al,* 1983; Ausubel, 1984)). The consensus sequence derived from this analysis is shown below :-

```
          -24              -12
5'-TGGYRYR- N4 - TTGCA -3'
```

The important nucleotides within this consensus sequence are the conserved GG doublet, found at position -25/-24 and the GC doublet found at position -13/-12. The spacing between these doublets is invariably 10bp, although the position of these doublets with respect to the transcription start site can vary. The homology of the *xylCAB* promoter region of the TOL plasmid, with this consensus sequence lead to the demonstration that this promoter was indeed σ^{54} dependent (Dixon, 1986). This provided the first indication that these promoters had a much more extensive usage than simply genes involved in nitrogen regulation and fixation .

The sequences of several σ^{54} dependent *Pseudomonas* promoters have now been determined and the following *Pseudomonas* specific consensus sequence has been proposed (Deretic *et al*, 1989):

$$\overset{G}{T}NNNT\overset{-24}{GG}CAT\overset{T}{G}NtaaTT\overset{-12}{GC}T\overset{A}{T}GNNAaA$$

This sequence varies slightly from the *nif* derived consensus but maintains the -24 GG and -12 GC doublets. For examples of *Pseudomonas* σ^{54} dependent promoter sequences see Table 1.2.

In general, when σ^{54}- holoenzyme binds to its cognate promoter element a closed complex is formed, which is transcriptionally non-productive (Sasse-Dwight and Gralla, 1988; Popham *et al*, 1989). The process of open complex formation requires the presence of a transcriptional activator protein which catalyses this isomerisation reaction (Popham *et al*, 1989). This mechanism of regulation is markedly different from the transcription initiation process at σ^{70} type promoters where σ^{70}-holoenzyme is capable of forming open complexes in the absence of ancillary proteins (Hellman and Chamberlin, 1988). The mechanism of regulation of several σ^{54} - dependent promoters has been well characterised and is described below.

b) Regulation of glutamine synthetase production in *E.coli*

When the supply of ammonia, the preferred nitrogen source, becomes restricted, enteric bacteria respond by increasing the expression of a number of operons (Magasanik, 1982). These nitrogen regulated (Ntr) operons encode products that facilitate firstly the assimilation of low concentrations of ammonia by means of the glutamine synthetase - glutamate synthase pathway (Reitzer and Magasanik, 1987) and then the utilisation of alternative sources of nitrogen.

The heart of the global nitrogen control system is the *glnALG* operon, which is best characterised in *E.coli* (Figure 1.2). *glnA* encodes glutamine synthetase, *glnL (ntrB)* encodes the regulatory gene NR_{II}, and *glnG (ntrC)* encodes the regulatory gene NR_I (Backman *et al*, 1981; Chen *et al*, 1982; Mcfarland *et al*, 1981). This operon has three promoters: *glnAp1* , with a transcriptional start site located 187 base pairs upstream from the *glnA* start codon; *glnAp2* with a transcriptional start site 73bp upstream from the *glnA* start codon; and *glnLP*, with a transcriptional start site 33bp upstream from *glnL*. (Reitzer and Magasanik, 1985; Ueno-Nishio *et al*, 1984). Located between the end of *glnA* and the *glnLp* promoter is a rho-independent transcription terminator and approximately three of every four *glnA* transcripts terminate here (Magasanik, 1988).

The promoters *glnAp1* and *glnLp* have the characteristic -10 and -35 regions recognised by σ^{70}-holoenzyme and are responsible for a basal level of expression of all three genes under conditions of ammonia excess. The initiation of transcription from these promoters is negatively regulated by NR_I and NR_I-phosphate. This repression results from the ability of NR_I to bind to a sequence, which overlaps the -35 region of *glnAp1*. The consensus sequence recognised by NR_I is $GCAN_6TCGCT$ (Reitzer and Magasanik, 1986; Ueno-Nishio *et al*, 1984)). This repression occurs under nitrogen excess growth conditions and limits the synthesis of glutamine synthetase and NR_I itself. The initiation of transcription from *glnAp1* is additionally regulated by CAP and cAMP (Reitzer and Magasanik, 1987).

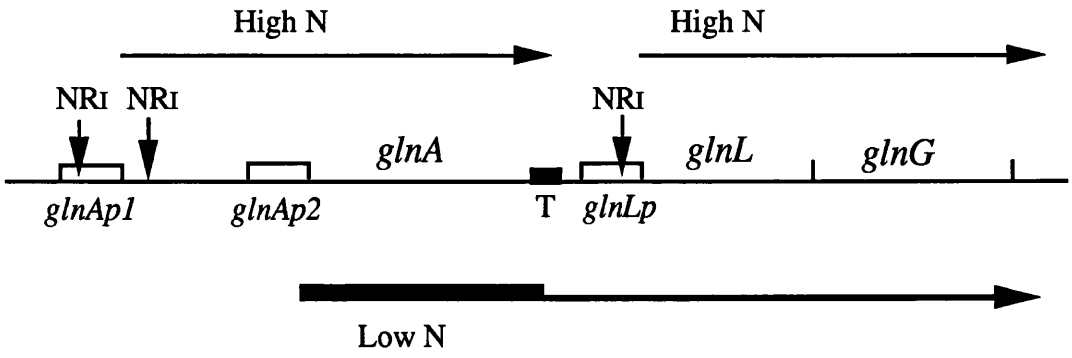


Figure 1.2. Organisation of the *glnALG* operon of *E.coli* . *glnAp1*, *glnAp2* and *glnLp* are the three promoters. The shaded block marked T represents a *rho* -independent terminator . *glnA* encodes glutamine synthetase, *glnL* encodes NRII and *glnG* encodes NRI. The vertical arrows indicate NRI binding sites. The horizontal arrows indicate the extent of transcription under ammonia excess growth conditions (High N) or limiting ammonia (Low N). Redrawn from Magasanik (1988)

The *glnAp2* promoter is a σ^{54} dependent promoter and is responsible for the increased expression of *glnA, glnL* and *glnG* under nitrogen limiting conditions. The activation of *glnAp2* involves a regulatory cascade, involving four components: (i) a uridylyltransferase/uridylyl-removing enzyme, (ii) P_{II}, the product of *glnB*, (iii) NR_{II}, an NR_I-kinase/NR_I-phosphatase, iv) NR_I, a transcriptional activator/ repressor (reviewed in Magasanik, 1988).

A reduction in the intracellular concentration of ammonia leads to a rapid drop in the intracellular concentration of glutamine , followed by a rise in that of 2-ketoglutarate, whose conversion to glutamate requires glutamine or ammonia. Consequently the ratio of 2-ketoglutarate: glutamine is increased. This ratio is the environmental signal by which the Ntr system is activated. 2-ketoglutarate activates the uridylylation of the enzyme P_{II} (encoded by *glnB*) and glutamine stimulates the deuridylylation of P_{II} -UMP by uridylyl-removing enzyme/uridylyltransferase. Under nitrogen excess conditions, PII stimulates the phosphatase activity of NR_{II} , which in turn leads to dephosphorylation of NR_I. Under nitrogen limiting conditions, P_{II}-UMP is inactivate, which allows the bifunctional protein NR_{II} to phosphorylate NR_I (Reitzer and Magasanik, 1987). This cascade is summarised in Figure 1.3

The phosphorylated form of NR_I activates transcription from *glnAp2* as follows: σ^{54}- holoenzyme forms a stable closed complex at *glnAp2* which can be detected *in vitro* (Popham *et al,* 1989). This complex is unable to initiate transcription. NR_I catalyses the isomerisation of closed complex to open complex, and allows the subsequent initiation of transcription. This isomerisation reaction is ATP dependent (Popham *et al,* 1989).

Optimal transcription from *glnAp2* requires the binding of NR_I to specific sites upstream of the start site of transcription (Ninfa *et al,* 1987; Popham *et al,* 1989) and these binding sites are illustrated in Figure 1.4. The contacts made by NR_I *in vivo* and *in vitro* indicate that NR_I binds with high affinity to sites 1 and 2 and with moderate affinity to site 3. NR_I also binds weakly to sites 4 and 5 *in vitro* (Ninfa *et al,* 1987) but not *in vivo* (Sasse-Dwight and Gralla, 1988). NR_I can

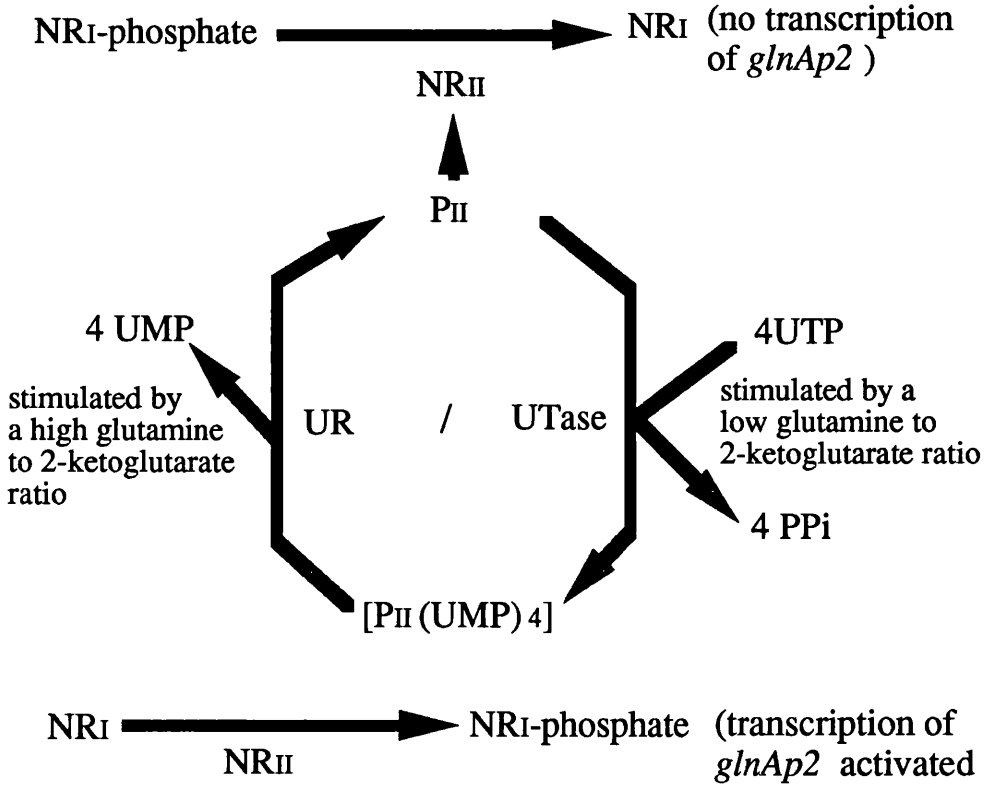


Figure 1.3. The regulatory cascade leading to activation/ repression of NRI. UR refers to the uridylyl group removal activity of *glnD*, UTase refers to the uridylyl transferase activity of the *glnD* gene product. PII stimulates the phosphatase activity of NRII, resulting in the dephosphorylation of NRI, which is then unable to stimulate transcription from *glnAp2*. Uridylylation of PII, prevents the stimulation of NRII as a phosphatase and allows NRII to phosphorylate NRI, which then activates transcription from *glnAp2*. Redrawn from Magasanik (1988).

Figure 1.4. Binding sites for NR_I and RNA polymerase at the *glnAp2* promoter of *E.coli*. The start site for transcription from *glnAp2* is designated +1. The high affinity NR_I binding sites, 1 and 2, the lower affinity site 3 and the very low affinity sites 4 and 5 are indicated. The *Met* indicates the start of the *glnA* coding sequence. Redrawn from Reitzer *et al* (1989). The conserved -24 GG and -12 GC doublets of *glnAp2* are shown in bold.

stimulate transcription from *glnAp2* without the high affinity sites 1 and 2, but expression is only 15% of the maximal level and requires a high concentration of NR_I. The high affinity sites 1 and 2 can be moved up to 1000bp upstream from *glnAp2* without diminishing the ability of NR_I to stimulate transcription (Reiztzer and Magasanik, 1986). In this respect the NR_I binding sites resemble enhancer sequences in eukaryotic cells. Under nitrogen limiting conditions, approximately 70 molecules of NR_I are produced in the cell. This high level of NR_I allows the activation of other nitrogen regulated operons in the cell, which do not necessarily possess NR_I binding sites (Magasanik and Neidhardt, 1987)

Recently it has been shown that tightly bound NR_I can activate transcription *of* the *glnAp2* promoter, from either side of the DNA helix, when the binding site is separated from RNA polymerase by at least 30bp. However activation from a partial NR_I binding site only occurs from one side of the DNA. The activation from the two high affinity binding sites is most effective when both sites are on the same side of the DNA helix. These results led to the hypothesis that NR_I activation of σ^{54}-holoenzyme involves a direct interaction and thus requires looping of the DNA to facilitate this contact (Reitzer *et al*, 1989).

c) The *nif* promoters - occupancy of promoters by σ^{54}- holoenzyme and the requirement for upstream activator sequences.

The *nif* gene cluster of *Klebsiella pneumoniae* consists of 20 genes arranged in eight operons. The *nif* promoters were amongst the first promoters shown to be transcribed by σ^{54}-holoenzyme (Gussin *et al*, 1986). The expression of the two regulator genes *nifL* and *nifA*, which form an operon is dependent on the transcriptional activator NR_I which stimulates transcription from the *nifLA* promoter under nitrogen limiting conditions (Espin *et al*, 1982).

NifA is a transcriptional activator protein, which stimulates transcription from the other *nif* gene promoters. With both the *nifH* and *nifU* promoters it has been demonstrated that NifA catalyses open complex formation (Cannon *et al*, 1990; Morett and Buck, 1989) . Upstream activator sequences (UAS) have been

identified in *nif* promoters, which are generally located around 100bp upstream from the transcription initiation site and have the conserved sequence TGT-N_{10}-ACA (Buck *et al*, 1986). The UAS is a binding site for NifA and the DNA binding region of NifA resides in the carboxy terminus (Morett and Buck, 1988; Morett *et al*, 1988). It is thought that NifA binding to the UAS increases its local concentration, and orientates it appropriately for interaction with σ^{54}-holoenzyme by DNA loop formation (Buck *et al*, 1987). *nifL* is a repressor of *nifA* and is thought to diminish the affinity of NifA for upstream activator sequences (Morett *et al*, 1990).

Recently, it has become clear that there are two classes of σ^{54}-dependent promoter, which differ in their requirement for an upstream activator sequence (Buck and Cannon, 1989; Morett and Buck, 1989). These observations have arisen from the analysis of the *nifH* promoter of *Klebsiella pneumoniae* and *Rhizobium meliloti.*

The *nifH* promoter of *K. pneumoniae* shows a strict dependence on an UAS, with NifA bound, for activation. *In vivo* footprinting indicates that the σ^{54}-holoenzyme interaction with this promoter is very weak, and stable closed complexes are not formed (Morett and Buck, 1989). In contrast, mutations in this promoter between positions -17 and -15 to give the sequence TTT allow stable closed complexes to be detected and result in a promoter which can be partially activated by a mutant form of NifA unable to bind to DNA (Buck and Cannon, 1989; Morett and Buck, 1989). These mutations generate a promoter with more similarity to the consensus *nif* promoter sequence. NifA mediated activation of the the *nifH* promoter of *Rhizobium meliloti* is not dependent on a UAS and stable closed complexes can be detected *in vivo.* This promoter also has the sequence TTT at positions -17 to -15, as does the consensus *nif* promoter sequence.

There thus appears to be one class (strong) of promoters at which σ^{54}-holoenzyme can form stable closed complexes and the stimulation of closed to open complex by the activator protein does not require binding to an upstream activator sequence. The *glnAp2* promoter of *E.coli* falls into this class since

stable closed complexes can be detected and it can be activated by NR_I in the absence of upstream binding sites (at high NR_I concentrations)(Ninfa *et al,* 1987; Popham *et al,* 1989).

With the second class of promoter (weak), the interaction of σ^{54}-holoenzyme with the promoter appears to be weak and in this case there is a strong dependency for the transcriptional activator to be bound to an UAS. This in turn ensures a local concentration of activator near the σ^{54}-holoenzyme binding site, which might stabilise the interaction of σ^{54}-holoenzyme with the promoter before open complex formation. Alternatively this may ensure that all weak interactions between σ^{54}-holoenzyme and the promoter, are productive leading to open complex formation (Morett and Buck, 1989). Analysis of the *nif* promoter sequences indicates that sequences with high homology to the consensus tend to fall into the first class i.e form stable closed complexes, whereas sequences which diverge from the consensus, e.g. *K. pneumoniae nifH,* fall into the second class.

d) A new paradigm in gene regulation - sensory pairs of proteins which respond to environmental stimuli

In recent years it has been recognised that several global regulatory networks in bacteria are controlled by two component regulatory systems which are responsive to environmental stimuli (Ronson *et al,* 1987b). The first component of such systems is a sensory protein which responds to an environmental signal. This sensory protein then modulates the activity of a regulator protein, which is generally a transcriptional activator, and stimulates expression of the relevant genes.

The best characterised two component systems include NR_I and NR_{II} (see section 1.3.4b), involved in the control of nitrogen metabolism; EnvZ (sensor) and OmpR (activator) which control the transcription of the porin genes in *E.coli* (Stock *et al,* 1990) ; and CheA/CheY involved in chemotaxis (Stock *et al,* 1990). In each case the sensory protein is a kinase that uses ATP to phosphorylate itself at a histidine residue. The phosphoryl group from the histidine protein kinase is

transferred to an aspartic side chain within a conserved domain of the transcriptional regulator (Aiba *et al,* 1989; Stock *et al,* 1988; Sanders *et al,* 1989) and this modulates its' activity.

A comparison of the sequences of a large number of sensor proteins indicates that there are two conserved domains. A conserved C-terminal domain of approximately 100 amino acids (Ronson *et al,* 1987b) and a conserved domain surrounding the phosphorylated histidine. Analysis of CheA indicates that deletion of the conserved C-terminal domain from phosphorylated CheA still allows this protein to donate the phosphate group to CheY, yet the protein is not capable of autophosphorylation at the histidine residue (Hess *et al,* 1988). This indicates that the conserved C-terminal domain of the sensory proteins is responsible for ATP binding and histidine phosphorylation.

The transcriptional regulator proteins all share a homologous N-terminal domain of approximately 130 residues (see Figure 1.5). The three dimensional structure of one of these regulators CheY has recently been determined (Stock *et al,* 1989) and it has been shown that the highly conserved aspartate residues (Asp13, Asp57) together with Asp12 are clustered in an acid pocket which is the site of aspartyl phosphorylation, mediated by the sensor protein. (Stock, 1988; Sanders *et al,* 1989). The high conservation of sequences within the N-terminal domain of the regulator proteins would suggest they adopt a similar basic structure to that of CheY (Stock *et al,* 1990).

The conservation of domains within bacterial activator proteins is not restricted to those which form one half of a two component sensory pair as described above. For example, the transcriptional regulator XylR, from the TOL plasmid (Inouye *et al,* 1988) has two characteristic regions that are homologous to NtrC (NR_I) (Buikema *et al,* 1985) and NifA (Drummond *et al,* 1986) . The C-terminal region of all three proteins contains a helix-turn helix motif , involved in the DNA binding activity of these proteins. The central region of XylR corresponds to the σ^{54}-interacting domain of NtrC and NifA (Nakazawa *et al,* 1990). This central

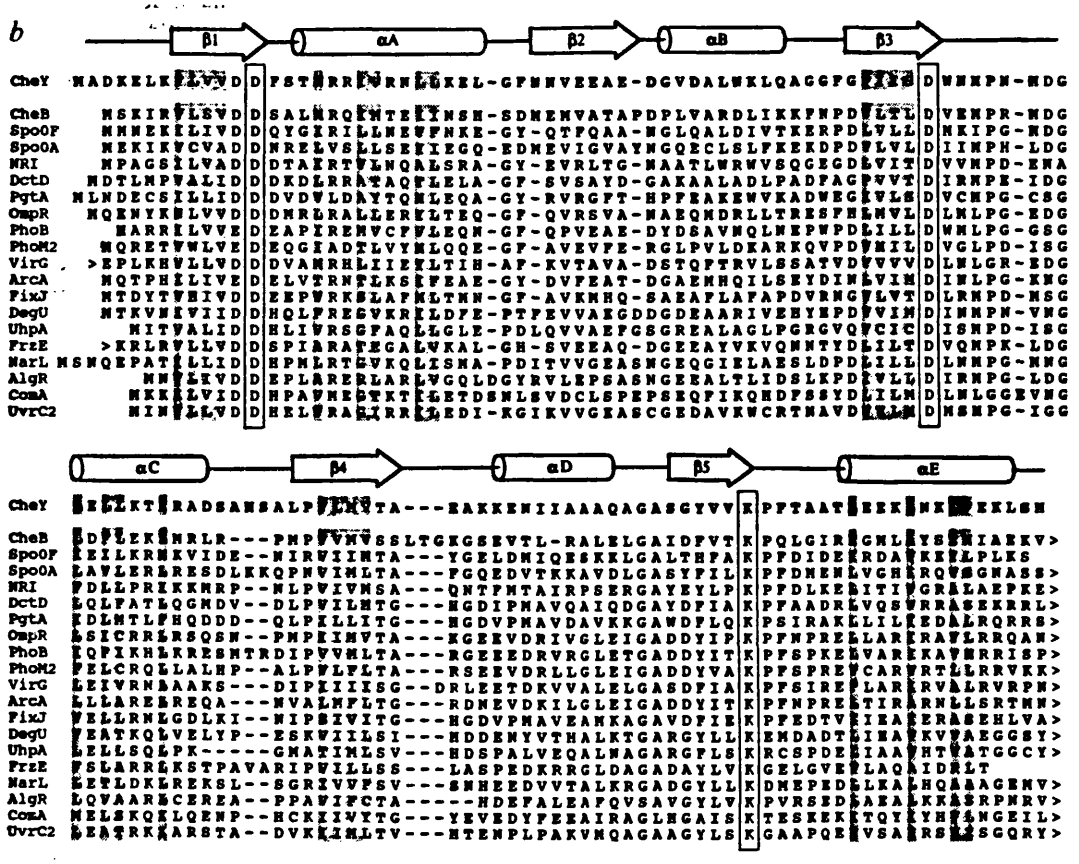


Figure 1.5. Alignment of homologous sequences of response regulator proteins with the known structural elements of CheY. The shaded areas represent the regions which form the hydrophobic core in CheY. The three residues that are highly conserved in the response regulators (transcriptional activators) are boxed (Asp 13, Asp57, Lys 109). Taken from Stock *et al* (1990).

domain in NtrC and NifA contains an ATP binding site and a point mutation has been constructed within the ATP binding motif in NtrC which abolishes its activator function at *glnAp2* (Drummond *et al*, 1990). This is consistent with the findings of Popham *et al* (1989) who demonstrated that the closed to open complex isomerisation reaction was ATP dependent.

Other families of bacterial activator proteins have been identified with conserved domains: i) the AraC family, which have conserved C-terminal domains. This family includes the *Pseudomonas* TOL plasmid encoded XylS protein, a postive regulator for the catabolism of benzoate . Eight proteins have been identified in this family, at least six of which are involved in the catabolism of carbon sources (Ramos *et al*, 1990). ii) The LysR family of bacterial activators, which have a conserved helix turn helix motif at the amino terminus (Henikoff *et al*, 1988) as well as several other common features.

The identification of sensory pairs of proteins responding to environmental stimuli, and the use of a similar chemical reaction to transfer information would suggest a common ancestral pair of genes, or protein domains, which have been adapted to respond to wide ranging environmental cues. Similarly, the identification of superfamilies of bacterial activator proteins often stimulating similar metabolic functions, would imply that within each family there was a common ancestral gene.

1.4 Positive control of gene expression by transcription antitermination

1.4.1 Introduction

Several proteins that have been shown to regulate transcription termination in prokaryotic systems have in common a requirement to recognise and bind to specific sequences in mRNA. For example, the *E.coli* termination factor rho, binds to C-rich regions in nascent RNA in order to subsequently cause the transcription complex to dissociate from the DNA template (Lowery and Richardson, 1977). The N protein of bacteriophage λ is the best characterised example of a transcription

antiterminator that functions by promoting the formation of a ribonucleoprotein complex composed of N protein and several host proteins, assembled around a sequence specific hairpin structure in the mRNA. This complex associates with the transcribing RNA polymerase and remains bound to it, allowing the RNA polymerase to read through multiple rho-dependent and rho-independent terminators (Roberts, 1988; Lazinski *et al,* 1989). With the *trp* operon of *E.coli*, expression is regulated by an attenuation mechanism, in which translation of an RNA leader sequence by ribosomes determines whether or not an RNA terminator structure will form (Yanofsky, 1988). Thus there appears to be at least two ways in which RNA binding proteins can regulate transcription termination. Firstly there are proteins like λ N which , upon binding RNA, directly interact with the transcription apparatus, modifying it, in such a way that the complex reads through downstream terminators. Secondly, with attenuation, the ribosome influences termination by its effect on RNA secondary structure, and under certain circumstances prevents terminator structures forming in the mRNA.

There are two well characterised chromosomally located catabolic operons which use antitermination to regulate gene expression (*bgl and sac*) and do not appear to require multiple host factors for the antitermination process as does the λ N system.

1.4.2 The *bgl* operon of *E.coli*

The *bgl* operon of *E.coli* encodes three genes, *bglG* (transcription antiterminator, *bglF* (negative regulator/ sugar permease) and *bglB* (structural gene), which are required for the utilisation of aromatic β-glucosides such as salicin (Mahadevan & Wright, 1987; Schnetz *et al,* 1987). The organisation of the operon is illustrated in Figure 1.6. The operon is cryptic in wild type cells, but a variety of mutations, mostly *cis* acting can lead to inducible expression . The most frequently encountered activating mutations are insertions of either IS1 or IS5 immediately upstream of the main operon promoter PO (see Fig, 1.6) (Reynolds *et al,* 1981; Lopilato and Wright, 1990). The mechanism of activation of PO by IS1 or IS5 remains unclear since it is not dependent upon transcription from the insertion sequences themselves (Saedler *et al,* 1972; Schnetz and Rak, 1988).

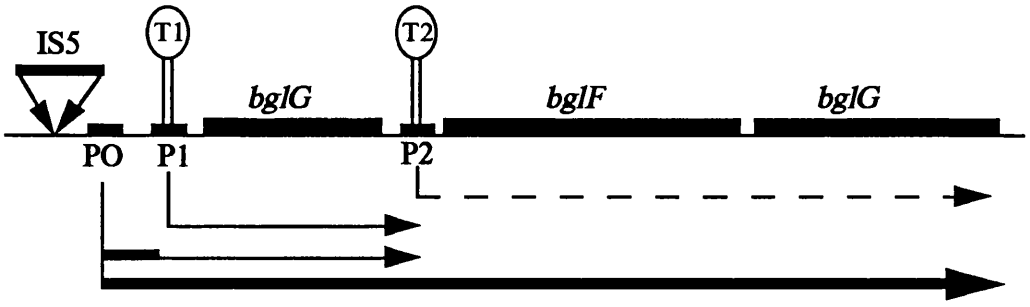


Figure 1.6. Organisation of the *bgl* operon of *E.coli.* The operon is cryptic in wild type cells and requires either IS1 or IS5 insertion upstream of PO for activation. T1 and T2 are two rho-independent transcription terminators. P1 and P2 are weak promoters which lie within the terminator sequences T1 and T2. *bglG* is a transcription antiterminator which positively regulates expression of the operon at T1 and T2. *bglF* encodes a negative regulator of the operon which regulates the activity of BglG. *bglB* encodes the structural enzyme, β-glucosidase. P1 and P2 allow a basal level of expression of all three genes under non-inducing conditions and under inducing conditions PO drives high level expression of the entire operon.

Once activated, expression of the operon is inducibly regulated by β-glucosides and is subject to catabolite repression.

Transcription from the activated PO is constitutive, but in the absence of inducer most transcripts terminate at the rho-independent terminator T1 located in the *bglG* leader sequence (Figure 1.6). Downstream of *bglG* is a second terminator (T2). Under inducing conditions the *bglG* gene product acts as a transcription antiterminator at these two terminators, allowing transcription of *bglG, bglF* and *bglB*. The system contains two additional promoters, P1 and P2, located within T1 and T2 respectively, which ensure basal levels of expression of *bglG* and *bglF* under non-inducing conditions. *bglF* is a negative regulator of the operon and also plays a direct role in the uptake of β-glucosides, by encoding enzyme II^{bgl} of the phosphoenolpyruvate dependent phosphotransferase system (PTS) (Bramley & Kornberg, 1987a).The PTS also comprises the cytoplasmic phosphoproteins enzyme I and HPr, which are not sugar specific. In this system, a phosphate group is transferred from phosphoenolpyruvate via enzyme I to a histidine residue of HPr and then onto the histidine residue of enzyme II^{bgl} (*bglF*).

Under non- inducing conditions the *bglF* gene product functions as a negative regulator of *bglB* expression by phosphorylation of BglG. BglG is thus inactivated with respect to antitermination activity. Under inducing conditions, the phosphate group is transferred from enzyme II^{bgl} to the incoming β-glucoside and BglF dephosphorylates BglG (Amster-Choder *et al,* 1989; Bramley and Kornberg, 1987b). The native form of BglG is active as a transcription antitermination factor and operon induction follows. This mechanism is illustrated in Figure 1.7.

Homology searches of the control regions of the *bgl* system identified two blocks of sequence immediately upstream and partially overlapping T1 and T2 (Schnetz *et al,* 1987). Deletions within these sequences drastically reduced the efficiency of antitermination by BglG (Schnetz and Rak, 1988). This region was further implicated in the antitermination process by the findings of Mahadevan and Wright (1987) that a 6bp insertion, 12bp upstream of the termination loop,

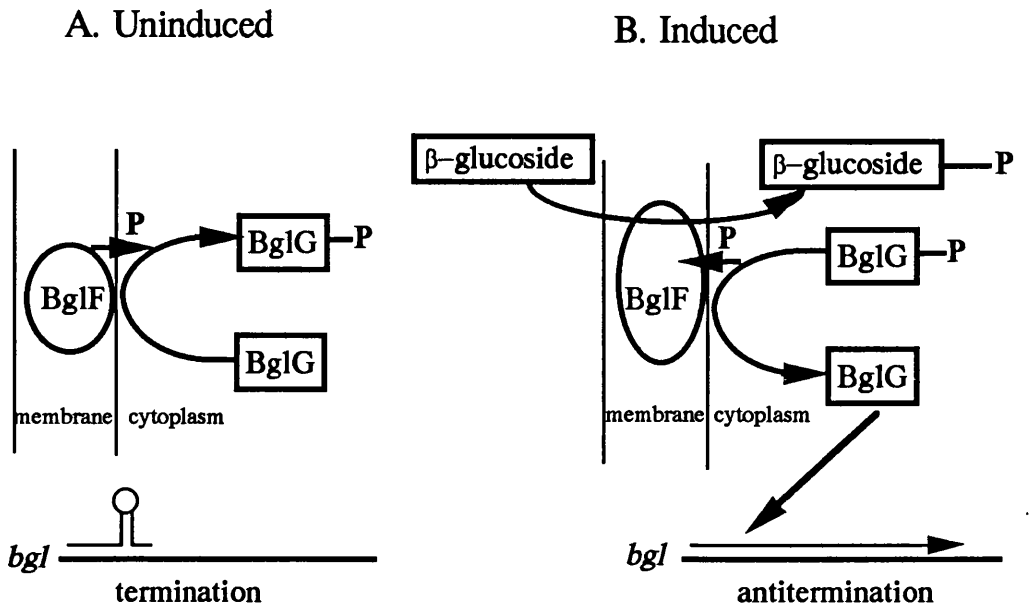


Figure 1.7. The regulation of BglG activity by BglF. Under non-inducing conditions, BglF phosphorylates BglG, inhibiting its antitermination activity. Under inducing conditions, BglF dephosphorylates BglG, activating it, and also phosphorylates incoming β-glucosides. Redrawn from Amster-Choder *et al* (1989).

rendered the system non-inducible. The most recent studies using purified mRNA from the leader region and purified BglG have shown that the protein binds to a specific sequence in the mRNA. Mutational analysis showed that the recognition was partly sequence specific but primarily a specific secondary structure recognition in the RNA. These findings led to the proposal that BglG binding to a site which overlaps the terminator, prevents formation of the terminator structure in the nascent RNA and thus allows transcription to proceed unhindered (Houman *et al*, 1990).

This mechanism is functionally distinct from the antitermination mechanism of λ N since it does not involve a direct interaction with RNA polymerase, and in many respects is more like an attenuation mechanism where the formation of a terminator structure is prevented by the ribosome.

1.4.3 The *sac* genes of *Bacillus subtilis*

Bacillus subtilis produces an extracellular levansucrase encoded by the *sacB* gene. The synthesis of this enzyme is induced by sucrose. Mutations which render *sacB* expression constitutive were mapped in three different loci, *sacR, ptsI* and *sacX-sacY* (Gonzy-Treboul & Steinmetz, 1987; Lepesant *et al*, 1972). *sacR* is the *cis*-regulatory region upstream of the *sacB* gene. It contains the promoter for *sacB,* followed by a transcription terminator, followed by the *sacB* coding sequence (Steinmetz & Aymerich, 1986) . In the leader sequence between the promoter and the transcription terminator are sequences homologous to the boxA and boxB motifs found in the *bgl* operon leader sequence of *E.coli* which have been shown to form the binding site for the transcription antiterminator *bglG* (Figure 1.10) (Schnetz *et al*, 1987; Houman *et al*, 1990). Point mutations or deletions which map within the transcription terminator, render *sacB* expression constitutive (Steinmetz & Aymerich, 1986).

The *ptsI* gene encodes enzyme I of the PTS, involved in the uptake and phosphorylation of a large number of sugars (for review, see Postma & Lengeler, 1985) and mutations in this gene render *sacB* expression constitutive (Gonzy-Treboul & Steinmetz, 1987). *sacX* and *sacY* form an operon (Zukowski *et al*, 1990) and

sacX is homologous with several sucrose-specific permeases of the PTS (Zukowski *et al*, 1990). The *sacY* gene, downstream of *sacX* codes for the positive regulator of *sacB* expression and causes transcription antitermination in the *sacB* leader region (Crutz *et al*, 1990). The *sacY* gene shows homology with *bglG*, the transcription antiterminator in the *bgl* operon of *E.coli* (Steinmetz *et al*, 1989).

The current model for the induction of *sacB* is as follows: The SacX protein is phosphorylated by the PTS (enzyme I, HPr, and probably a sucrose specific enzyme III). In this state it inhibits the SacY transcription antiterminator, possibly by phosphorylation. This prevents antitermination and transcription of the *sacB* gene. The presence of external sucrose results in transport and concomitant phosphorylation by *sacX*. The resulting dephosphorylation of SacX relieves SacY inhibition, permitting antitermination and transcription of *sacB* (Crutz *et al*, 1990).

These two catabolic systems, both positively regulated by a transcription antitermination system, show homology at the DNA sequence level despite their evolutionary divergence.

1.5 The amidase system of *Pseudomonas aeruginosa*

1.5.1 The early genetic and physiological studies

P.aeruginosa strain PAC1 produces an aliphatic amidase which enables growth on acetamide and propionamide as the sole source of carbon and nitrogen (Kelly & Clarke, 1962). The physiological reaction of the wild type amidase is the hydrolysis of amides, however *in vitro* amidase can carry out a number of other reactions which are summarised in Table 1.3. Synthesis of the enzyme is induced by a limited range of short chain aliphatic amides, however, inducer and substrate specificities differ (Kelly & Clarke, 1962). Certain amides can repress amidase expression for example butyramide (Brammar & Clarke, 1964). Gratuitous inducers have been found as have some non-inducing substrates. The latter have proved useful in isolating constitutive mutants (Brammar *et al*, 1967).

Table 1.3. Amidase Reactions.

(1) Amide hydrolysis

$R.CO.NH_2 + H_2O = R.CO_2^- + NH_4^+$

(2) Transferase activity (amide as substrate)

$R.CO.NH_2 + NH_2.OH + H^+ = R.CO.NH.OH + NH_4^+$

(3) Transferase activity (acid as substrate)

$R.CO_2^- + H^+ + NH_2.OH = R.CO.NH.OH + H_2O$

(4) Ester hydrolysis

$R.CO_2.\ R' + H_2O = R.CO_2^- + H^+ + R'\ OH$

(5) Transferase activity (ester as substrate)

$R.CO_2.\ R' + NH_2.\ OH = R.CO.NH.OH + R'.\ OH$

From McFarlane *et al.* (1965)

Initial transduction analysis showed that the structural gene for amidase *amiE* (defined by Ami$^-$ mutations) and the regulatory gene *amiR* (defined by constitutive mutations) were closely linked (Brammar *et al*, 1967). *amiR* has been shown to be a positive regulator of amidase expression based on two main lines of evidence (Farin and Clarke, 1978). Firstly, mutant *P.aeruginosa* strains were isolated which had an amidase negative phenotype at 42°C but were amidase positive at 28°C. The temperature sensitive mutations were shown to be in the regulator gene since the strains produced the wild type, thermostable amidase. Secondly, several amidase negative mutants were isolated which reverted to give a variety of regulatory phenotypes. This suggested that the original Ami$^-$ mutations had been in the regulator gene and that *amiR* encoded a positive regulator.

Amidase synthesis is also subject to catabolite repression by succinate and other intermediates of the TCA cycle (Smyth and Clarke, 1975a,b), although the mechanism of catabolite repression in *P.aeruginosa* is poorly understood. Amidase expression in PAC strains does not appear to be regulated by nitrogen availability (Potts and Clarke, 1976), though Janssen *et al* (1982a) have demonstrated nitrogen regulation of amidase synthesis in *P.aeruginosa* strain PAO1.

1.5.2 Cloning of the amidase genes

The amidase genes were initially cloned from *P.aeruginosa* strain PAC433, a constitutive, up-promoter mutant, resistant to both catabolite repression and repression by butyramide (Smyth & Clarke, 1975b). The genes were cloned into the bacteriophage lambda cloning vector λ722 to generate λ-*ami* (Drew *et al*, 1980). Recombinant bacteriophage containing the amidase genes were detected by enhanced growth of *E.coli* around the recombinant plaques on minimal media containing acetamide as the sole nitrogen source. Subsequently a 5.3kb *Hin*dIII-*Sal*I fragment from λ-ami was subcloned into pBR322 to generate plasmid pJB950. The *amiE* gene was found to lie close to the *Hin*dIII target of the fragment by comparison of the DNA sequence derived from saturation restriction mapping with the N-terminal amino acid sequence of amidase (Clarke *et al*, 1981). Complementation studies using the amidase structural gene on a broad host range plasmid in *P. aeruginosa* amidase mutant strains showed transcomplementation by the chromosomally located

regulator gene and these studies confirmed the positive control model for amidase expression (Drew, 1984). Recently the amidase genes from the wild type inducible strain PAC1 have been cloned into the λ vector L-47 and the homologous 5.3kb *Hin*dIII-*Sal*I fragment containing the amidase genes, subcloned into pBR322 to generate plasmid pAS20 (Wilson and Drew, 1991). This plasmid is identical in structure to pJB950 except it contains wild type genes, as opposed to the constitutive genes in pJB950. The DNA sequence and amino acid sequence of *amiE* (amidase) have been determined and the position of some of the AmiE substrate specificity mutations have been identified (Brammar *et al*, 1987; Ambler *et al*, 1987).

1.5.3 Characterisation of the *amiR* gene

Preliminary experiments indicated that sequences downstream of *amiE* in plasmid pJB950 were required for amidase expression in *E.coli* (Cousens and Drew, 1984). These results suggested that *amiR* was present on the 5.3kb *Hin*dIII-*Sal*I *Pseudomonas* DNA fragment present in pJB950. A series of *in vitro* constructed deletions were made in pJB950 and these experiments defined the *amiR* coding region to within a 950bp *Cla*I-*Xho*I fragment, some 2kb downstream of the *amiE* gene. The location of *amiE* and *amiR* in plasmid pJB950 is shown in Figure 1.8. Using subcloned DNA fragments, for transcomplementation studies in *P.aeruginosa amiR*$^-$ strains, the direction of transcription of *amiR* and *amiE* was shown to be the same. Attempts to locate the *amiR* promoter indicated that it lay within a 900bp region upstream of *amiR*. However, deletions spanning this region of DNA caused increased amidase expression (Cousens *et al*, 1987). These studies thus provided no evidence for the mechanism of expression of the *amiR* gene.

The DNA sequence of the 1.5kb *Xho*I *amiR* gene fragment from PAC433 was determined, and *amiR* was found to be 588bp long, encoding a protein product with a calculated molecular weight of 22.8kDa (Lowe *et al*, 1989). The deduced protein sequence for *amiR* was found to have no homology with any other sequences in the EMBL database.

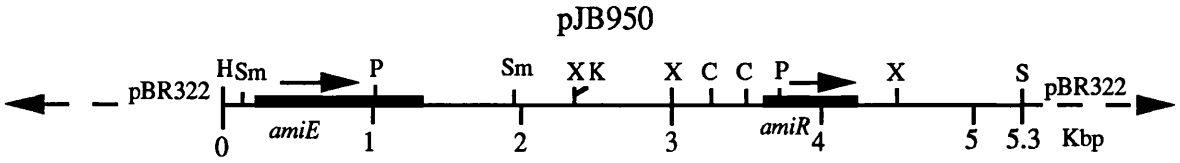


Figure 1.8 Restriction map of the *Hin*dIII-*Sal*I fragment of plasmid pJB950 (PAC433 genes), which was subcloned into the *Hin*dIII-*Sal*I targets of pBR322. The location and direction of transcription of *amiE* and *amiR* are shown by the arrows. Abbreviations for restriction enzyme targets are as follows: H= *Hin*dIII, P= *Pvu*II, X= *Xho*I, K= *Kpn*I, C= *Cla*I, S= *Sal* I, Sm = *Sma* I.

1.5.4 The transcriptional antitermination mechanism which regulates amidase expression

The DNA sequence between the *Hin*dIII target and the start codon for *amiE* has been determined and is shown in Figure 1.9. A consensus *E.coli* σ^{70} dependent promoter has been identified by sequence homology between residues 91 - 120. Downstream of this promoter element is a short open reading frame, followed by an axis of dyad symmetry which has the characteristics of a rho-independent transcription terminator. Downstream of this terminator is the start codon for *amiE* at position 262. By *in vitro* construction of a 10bp deletion within the terminator structure in plasmid pJB950 it was shown that amidase expression was increased seven fold from the pJB950 level of expression. Deletion of the *amiR* coding sequences had no effect on this elevated level of amidase expression. It was thus concluded that *amiR* exerted positive regulation of amidase expression by a transcription antitermination mechanism (Drew & Lowe, 1989).

The best characterised systems for transcription antitermination are the λ N and Q proteins. These proteins interact with RNA polymerase at defined utilisation sites and cause transcription antitermination. In the *amiE* leader sequence there are no sequences which have homology with these specific utilisation sites (*nut* and *qut*). However, Drew & Lowe, (1989) identified a region of DNA sequence upstream of the *amiE* terminator which was homologous with a region in the *bgl* leader sequence. The homologous region encompassed the site of the 6bp insertion shown to render the *bgl* system non-inducible (Mahadevan and Wright, 1987). Figure 1.10 shows an alignment of the leader sequences from *bgl* T1 and T2, the *sac* terminator and the *amiE* terminator region. The homology between these sequences, suggests that the antitermination mechanism is similar for each system.

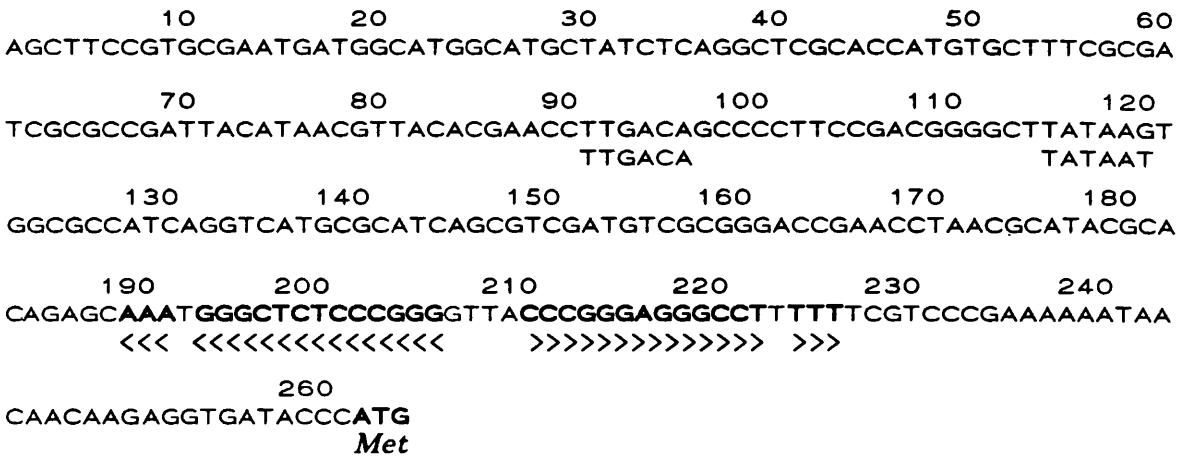


Figure 1.9. The DNA sequence upstream of *amiE*. The sequence runs from the *Hin*-dIII target to the *amiE* start codon, indicated by *Met*. The *E.coli* consensus promoter sequence is shown at positions 91-96 (-35) and 115-120 (-10), below the *amiE* sequence. The bold sequence with <<<<. >>>>> underneath indicates the axis of dyad symmetry which forms the rho-independent transcription terminator. Redrawn from Drew and Lowe (1989). There is a short open reading frame in the leader region which runs from positions 136-243.

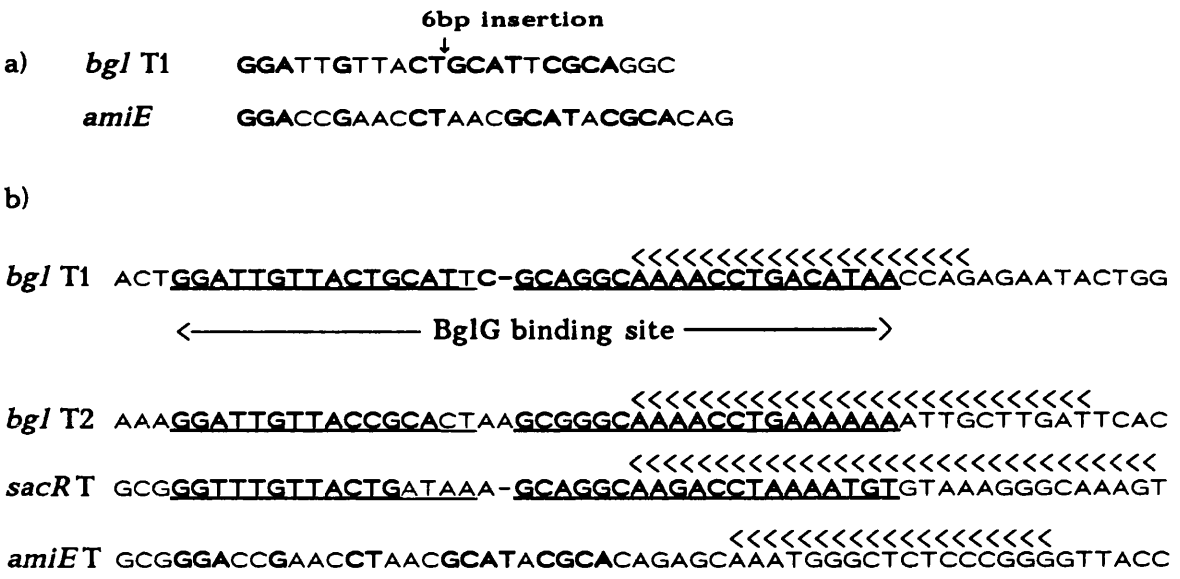


Figure 1.10. a) Alignment of the leader sequences upstream of *bgl* terminator 1 (T1), and the *amiE* terminator. The sequences in bold indicate homology. The arrow indicates the site of the 6bp insertion in the *bgl* sequence, which renders the system uninducible (Mahadevan and Wright, 1987).

b) Alignment of the leader regions for the two terminators of the *bgl* operon, the terminator for the *sac* operon of *Bacillus subtilis*, and the *amiE* terminator. The sequences in bold and underlined indicate the boxA and boxB motifs indentified by Schnetz *et al*, 1987). The <<<<<<<<<< symbol indicates the 5' side of the terminator structures (3' not shown). The BglG binding site has been defined at *bgl* T1 and is indicated by the arrows (Houman *et al*, 1990).

1.6 The aims of this investigation

Using the cloned amidase genes from PAC1 in plasmid pAS20 and from PAC433 in pJB950, the initial aims of the present study were to identify the *amiR* promoter and investigate the regulation of *amiR* expression. This would in turn allow a model for the induction process to be constructed. The recent cloning of the wild type amidase genes would greatly facilitate this analysis since *in vitro* constructed deletions and rearrangements of the genes in *E.coli* could be used to identify sequences involved in the induction process.

CHAPTER TWO MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacterial strains

The bacterial strains used in this study are listed in Table 2.1

2.1.2 Plasmids

The plasmids used in this study are listed in Table 2.2. Table 2.2(a) shows parental plasmids, Table 2.2(b) shows recombinant plasmids based on broad host, RSF1010 derived vectors and Table 2.2(c) shows recombinant plasmids based on pBR322, pUC18/19 or pACYC184 vectors.

I would like to thank the following people for providing strains and plasmids during this study: Chris Franklin, Michael Bagdasarian, John Ward, Karyn Ishimoto, Paul Brown, Ray Dixon and Norman Robillard.

2.1.3 Growth media

(a) Nutrient Broth:

i) *E.coli* strains were grown in L broth (Lennox, 1966).

Bacto tryptone	10 g/l
Bacto yeast extract	5 g/l
Sodium chloride	5 g/l
Glucose	1 g/l

pH 7.2

ii) *P. aeruginosa* strains were grown in Oxoid No. 2 nutrient broth. L-broth and nutrient broth were autoclaved at 15lb/in^2 for 15 minutes and stored at room temperature.

(b) Nutrient agar:

For plates and slopes 1.2% (w/v) Difco agar was added to either L-broth or Oxoid No. 2 nutrient broth. Solutions were autoclaved at 15lb/in^2 for 15

TABLE 2.1 Bacterial strains

a) *E.coli*

Strain	Genotype	Reference
JA221	*hsdR recA trp leu*	Clarke & Carbon (1978)
C600	*thr leu thi*	Harayama *et al* (1980)
JM101	*thi* Δ(*lac-proAB*) F'[*traD proAB*$^+$ *lacI*^{q} *lacZ* ΔM15]	Yanisch-Perron *et al* (1985)
ET8000	*rbs lacZ*::IS1 *gyrA hut* C_k^c	Dixon (1986)
ET8045[i]	*ntrA208*::Tn10	Dixon (1986)
ET8556[i]	*ntrC1488*	Dixon (1986)
ET8894[i]	Δ(*rha-glnA-ntrBC 1703*::Mucts62)	Dixon (1986)
UNF1963[i]	*ntrA208*::Tn10 *ntrC1488*	Dixon (1986)
HB101	*hsdS*$_B$ *recA pro rpsL20*	Rodriguez *et al* (1977)
DH5α	*endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ(*lacZYA-argF*)U169 L-Ø80 *dlacZ* ΔM15	Bethesda Research Laboratories

b) *P.aeruginosa*

Strain	Genotype	Amidase phenotype	Reference
PAC1	*amiE*$^+$*amiR*$^+$	Ind	Kelly & Clarke (1962)
PAC101	*amiE*$^+$*amiR1*	Con	Brammar *et al* (1967)
PAC111	*amiE*$^+$*amiR11*	Con	Brammar *et al* (1967)
PAC327	*amiE35 amiR33 crp7*	Ami$^-$	Brown (1969)
PAC433	*amiE114,120 amiR1*	Con CRP^R But^R	Smyth & Clarke (1975b)
PAC452	*amiΔ161*	Ami deletion	Day (1975)
PAC623	*amiE16 amiR223*	Ami$^-$	Farin & Clarke (1978)
PAK-SR	Sm^R mutant of PAK	Ind	Ishimoto & Lory (1989)
PAK-N1[ii]	*ntrA*	Ind	Ishimoto & Lory (1989)
PAC200	*amiE*$^+$ *amiR*$^+$*ntrA*	Ind	This study

i) These strains are derived from ET8000 and have the basic genotype of ET8000 in addition to those mutations shown.

ii) This strain is derived from PAK-SR and has the same basic genotype of PAK-SR in addition to those mutations shown.

Phenotype abbreviations: Ind, inducible; Con, constitutive; Ami$^-$, amidase negative; CRP^R, resistant to catabolite repression; But^R, resistant to butyramide repression.

Table 2.2 Plasmids

(a)

Plasmid	Size (kbp)	Markers	Reference
pBR322	4.4	Ap^R Tc^R	Bolivar *et al* (1977)
pUC18	2.7	Ap^R	Yanisch-Perron *et al* (1985)
pUC19	2.7	Ap^R	Yanisch-Perron *et al* (1985)
pBGS19$^-$	4.4	Km^R	Spratt *et al* (1986)
pACYC184	3.9	Cm^R Tc^R	Chang & Cohen (1978)
pTH10	60.4	Ap^R Km^R Tc^R	Harayama *et al* (1980)
pRK2013	-	Km^R	Figurski & Helinksi (1979)
pAS20	9.1	Ap^R Ami^+ Ind	Wilson & Drew (1991)
pJB950	9.1	Ap^R Ami^+ Con	Clarke *et al* (1981)
pDC5	6.1	Ap^R *amiE*$^+$	Cousens *et al* (1987)
pMM14	-	Km^R *ntrC*^{C}	Merrick (1983)
pTS174	-	Cm^R *xylR*$^+$	Inouye *et al* (1983)
pMC71A	-	Cm^R *nifA*^{C}	Buchanan-Wollaston *et al* (1981)
pKI11	-	Ap^R *ntrA::tet*	Ishimoto & Lory (1989)
pKT231	13.0	Sm^R Km^R	Bagdasarian *et al* (1981)
pKT240	12.5	Ap^R Km^R	Bagdasarian *et al* (1983)
pMMB66HE	8.9	Ap^R (Cb^R)	Fürste *et al* (1986)
pMMB66EH	8.9	Ap^R (Cb^R)	Fürste *et al* (1986)
Phage			
M13mp18	7.3	*laci' lacZ'*	Yanisch-Perron *et al* (1985)
M13mp19	7.3	*laci' lacZ'*	Yanisch-Perron *et al* (1985)

(b)

Plasmid	Markers
pSW11B	Cb^R
pSW12B	Cb^R
pSW13B	Cb^R *amiC*
pSW14B	Cb^R *amiC*
pSW15B	Cb^R *amiC*^{C}
pSW16B	Cb^R *amiC*^{C}
pSW35	Sm^R *amiR*
pSW40	Cb^R *amiR*
pSW41	Cb^R *amiC*
pSW42	Cb^R
pSW101	Sm^R Ami^+
pSW113	Cb^R *amiC*
pSW115	Cb^R *amiC*^{C}
pSW116	Cb^R *amiC*^{C}

c)

Plasmid	Markers
pSW1	Ap^R *amiE amiR*
pSW2	Ap^R *amiE amiR*
pSW3	Ap^R *amiE amiR*
pSW4	Ap^R *amiE amiR*
pSW5	Ap^R *amiE amiR*
pSW7	Ap^R *amiE*
pSW9	Ap^R
pSW10	Ap^R promoterless *amiE*
pSW11	Ap^R
pSW12	Ap^R
pSW13	Ap^R *amiC*
pSW14	Ap^R *amiC*
pSW15	Ap^R *amiC* constitutive
pSW16	Ap^R *amiC* constitutive
pSW20	Ap^R
pSW21	Ap^R
pSW24	Ap^R *amiR* expression vector
pSW26	Ap^R *amiC* expression vector
pSW27	Ap^R *amiR* expression vector
pSW36	Ap^R *amiE amiR*
pSW37	Ap^R *amiE amiR*
pAS25	Cm^R *amiE amiR*
pTM1	Cm^R *amiE*
pTM2	Km^R *amiR* expression vector

minutes and allowed to cool to approximately 55°C before addition of antibiotics if appropriate and pouring into 85mm vented plastic Petri dishes. For overlaying plates 0.6% (w/v) Difco agar was used in the above media. Poured plates were stored in an inverted position at 4°C.

(c) Minimal medium:

i) *E.coli* basal medium with nitrogen (Clarke & Laverack, 1983):

Na_2HPO_4	4.5 g/l
KH_2PO_4	2.0 g/l
$(NH_4)_2SO_4$	1.0 g/l
NH_4Cl	1.0 g/l
KNO_3	0.5 g/l
Na lactate	0.8% (v/v)
E.coli trace elements	1.0% (v/v)

pH 7.0-7.2

ii) *E.coli* basal medium without nitrogen (P. Laverack, personal communication

Na_2HPO_4	4.5 g/l	0.01 % L-tryptophan and
KH_2PO_4	2.25 g/l	0.01% L-leucine were added as
Na_2SO_4	2.5 g/l	nitrogen source.
NaCl	1 g/l	
E.coli trace elements	1.0% (v/v)	

pH 70.0-7.2

iii) *E.coli* trace elements solution (P.Laverack, personal communication):

Na citrate	40.0 g/l	$ZnSO_4.7H_2O$	0.02 g/l
$MgSO_4.7H_2O$	20.0 g/l	$Na_2B_4O_7.7H_2O$	" "
$FeSO_4.7H_2O$	2.0 g/l	$(NH_4)_6Mo_7O_{24}.7H_2O$	" "
$MnCl_2.2H_2O$	1.0 g/l	$CuSO_4.5H_2O$	" "
$CaCl.2H_2O$	1.0 g/l	$CoCl_2$	" "

Solutions were autoclaved at 15lb/in^2 for 15 minutes and stored at room temperature. Additional carbon sources (0.5% w/v final concentration) were added as appropriate after autoclaving.

iv) *Pseudomonas* basal medium with nitrogen (Brammar & Clarke, 1964):

K_2HPO_4	12.5 g/l
KH_2PO_4	3.8 g/l
$(NH_4)_2SO_4$	1.0 g/l
$MgSO_4.7H_2O$	0.1 g/l
Trace elements solution	0.5% (v/v)
pH 7.2	

v) *Pseudomonas* trace elements solution (Kelly & Clarke, 1962):

H_3BO_3	0.23g/l	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.022g/l
$ZnSO_4.7H_2O$	0.174g/l	$CuSO_4.5H_2O$	0.008g/l
$FeSO_4(NH_4)_2SO_4.6H_2O$	0.116g/l	$MnSO_4.4H_2O$	0.008g/l
$CoSO_4.7H_2O$	0.096g/l		

Solutions were autoclaved at 15lb/in^2 for 15 minutes and stored at room temperature. Carbon sources were added (1% w/v) as appropriate after autoclaving.

(d) Minimal agar:

For solid medium, 1.2% (w/v) Difco agar was added to the above minimal media.

2.1.4 Antibiotic selective media

Antibiotics were added as appropriate to nutrient or minimal media at the following final concentrations:

	E.coli	*P.aeruginosa*
Ampicillin	100 µg/ml	–
Carbenicillin	–	1000 µg/ml
Kanamycin	100 µg/ml	250 µg/ml
Streptomycin	100 µg/ml	500 µg/ml
Gentamycin	10 µg/ml	–
Tetracycline	10 µg/ml	100 µg/ml
Chloramphenicol	15 µg/ml	–

For plates, antibiotics were added at the working concentration immediately before pouring.

2.1.5 Dilution Buffer

The dilution buffer was that of Brammar *et al* (1967):

KH_2PO_4	3.0 g/l
Na_2HPO_4	7.0 g/l
NaCl	4.0 g/l
$MgSO_4.7H_2O$	0.2 g/l

pH 7.2

2.1.6 Phenol/Chloroform

Phenol was prepared by melting Analar grade phenol at 70°C then equilibrating with 1M Tris pH 8.0, followed by 0.1M Tris pH 8.0 until the pH of the aqueous phase was greater than pH 7.5. Phenol/chloroform solution was prepared in a 1:1 (v/v) ratio. The chloroform used was a 24:1 (v/v) chloroform:isoamyl alcohol mix.

2.1.7 Ribonuclease A

Pancreatic RNase A was dissolved at a concentration of 10 mg/ml in 10mM Tris.HCL pH 7.5 and 15mM NaCl. Contaminating DNase activity was destroyed by heating at 100°C for 15 minutes. The solution was allowed to cool slowly to room temperature before storage at -20°C.

2.1.8 Reagents

The majority of reagents used were of analytical grade, purchased from B.D.H or Fisons, with the following exceptions:

Acrylamide	Sigma
Agarose	Sigma
Ampicillin	Beecham
Bacto agar	Difco
Bacto tryptone	Difco

Bovine serum albumin	B.R.L
Biodyne A nylon membrane	Pall Ultrafine Filtration Co.
Carbenicillin	Sigma
DNA modifying enzymes	Anglian Biotechnology Ltd
DNA linkers	N.E.B
DNA sequencing kits	Pharmacia
Ethanol	James Burroughs Ltd
Ficoll 400	Pharmacia
Hybond N^+ nylon membrane	Amersham
IPTG	Pharmacia
Kanamycin	Sigma
Lactamide	Sigma
Lysozyme	Sigma
3MM paper	Whatman Ltd
NAP-5 columns	Pharmacia
Oligonucleotides	Peter Swann, Biochemistry Dept., UCL.
Ribonuclease A	Sigma
Radiochemicals	NEN, Sigma
Restriction endonucleases	Anglian Biotechnology Ltd
S1 nuclease	Boehringer
Streptomycin	Sigma
Universal sequencing primer	Pharmacia
Tetracycline	Sigma
X-gal	Northumbrian Biotech. Ltd
X-ray film	Fuji Photo Film Co.
Bacto yeast extract	Difco

2.2 Methods

Microbiological Methods:

2.2.1 Bacterial growth conditions

Bacteria were routinely grown in 20ml universal bottles containing 5ml of media on a reciprocating shaker. Individual colonies were isolated by streaking a loopful of a bacterial culture on an agar plate. For large scale growth of bacteria, conical flasks were used which were a minimum of five times the volume of the culture they contained. The majority of strains were grown at 37°C. *E.coli* strains carrying a temperature sensitive plasmid or phage were grown at 28°C.

2.2.2 Storage of bacterial strains

(a) Short term storage:

P. aeruginosa and *E.coli* strains were stored in agar stabs in half ounce bottles. *P. aeruginosa* strains were kept at room temperature and *E.coli* strains were stored at 4°C.

(b) Long term storage:

Both *E.coli* and *P. aeruginosa* strains were stored in 33% sterile glycerol at -70°C.

2.2.3 Measurement of bacterial growth

The density of liquid cultures was determined by measuring the absorbance in a Pye Unicam SP6 350 spectrophotometer against a medium blank. *E. coli* cultures were read at 450nm while *P. aeruginosa* cultures were read at 670nm.

2.2.4 Transformation of *E.coli*

The method used was based on that of Mandel and Higa (1970) and yielded 10^6 transformants per microgram of pBR322 DNA when used with *E.coli* strain JA221.

(a) **Preparation of competent cells**

50 ml of prewarmed L-broth in a 500 ml flask was inoculated with 1 ml of an overnight culture and the cells grown at 37°C with vigorous shaking. 0.5 ml aliquots were removed at various time intervals to determine the extent of growth by measurement of the OD_{450}. At OD_{450} of 0.5 the culture was chilled on ice for ten minutes and the cells collected by centrifugation in a Sorvall RC2-B centrifuge at 5000rpm for 10 minutes at 4°C. The bacterial pellet was resuspended in 25 ml of an ice cold solution of 0.1M $MgCl_2$ and the cells harvested as before. The bacterial pellet was then resuspended in 2.5 ml 0.1M $CaCl_2$ and stored on ice for one hour. Fresh competent cells were prepared for each transformation.

(b) **Transformation**

1 ng-1 µg of plasmid DNA in TE buffer was added directly to 250 µl of competent cells in a 5ml sterile plastic Falcon tube. The cells were incubated on ice for forty-five minutes, heat-shocked in a 37°C water bath for five minutes and then returned to ice for thirty minutes. 0.7 ml of L-broth (preheated to 37°C) was added and the cells incubated at 37°C for one hour. This incubation period allowed time for the bacteria to begin expressing antibiotic resistance genes.

(c) **Plating of transformed *E.coli***

200 µl of transformed cells were overlayed in 0.6% nutrient agar on selective antibiotic plates. The plates were incubated in an inverted position at 37°C overnight.

2.2.5 Mobilisation of plasmids from *E.coli* to *P.aeruginosa*

Plasmids were mobilised using either pTH10 a temperature sensitive derivative of the drug resistance plasmid RP4 (Harayama *et al*, 1980) in *E.coli* strain C600 or plasmid pRK2013 (Figurski & Helinski, 1979) in *E.coli* strain HB101.

(a) **Use of plasmid pTH10**

Plasmid pTH10 was transferred from *E.coli* strain C600 into *E.coli* strains carrying recombinant broad host range plasmids by plate mating. 0.1 ml of overnight cultures of donor and recipient cells were spotted onto a nutrient agar plate and incubated at 29°C in an upright position for 4 hours. 1.0 ml of dilution buffer was then added and the cells lifted off using a sterile glass spreader and pipette. The cells were pelleted in an Eppendorf centrifuge for two minutes at room temperature and washed twice with dilution buffer by resuspension and centrifugation. The final cell pellet was resuspended in 0.5 ml dilution buffer and serial dilutions plated out onto appropriate selective plates. The plates were incubated in an inverted position for two days at 29°C. Transconjugants were shown to contain both plasmids by antibiotic resistance screening. Resulting strains were then used as donors to mobilise the recombinant plasmids into *P.aeruginosa* strains by plate mating as described above. *P.aeruginosa* transconjugants were identified after growth on appropriate selective plates, which were incubated at 37°C to facilitate loss of the pTH10 plasmid. Restriction enzyme analysis of plasmid DNA isolated from transconjugants showed only the presence of the recombinant plasmid.

(b) **Use of plasmid pRK2013**

Plasmid pRK2013 in *E.coli* strain HB101 was used in triparental matings (Deretic *et al*, 1986) with *E.coli* JA221 containing the broad host range plasmid and the recipient *P.aeruginosa* strain. 0.1 ml of each of the three strains were mixed on a nutrient agar plate and incubated at 37°C for six hours. The cells were then lifted off the plate, washed and plated on the appropriate selective plates as described above. The presence of the recombinant plasmid in the *P.aeruginosa* strain was determined by restriction enzyme analysis of plasmid DNA isolated from the strain.

2.2.6 Transfection of M13 DNA

Competent JM101 cells were prepared as described previously. 1-300 ng of

ligated M13 DNA was added directly to 250 µl of competent JM101 cells and the mixture incubated on ice for forty-five minutes in a 5 ml Falcon tube. The cells were heat shocked in a 37° C water bath for 5 minutes and then returned to ice for ten minutes. 100 µl of L-broth was added to the cells and the mixture was held at 37°C until plated.

2.2.7 Plating of transfected cells

3 ml aliquots of 0.6% L-agar were dispensed into 5ml Falcon tubes. 15 µl of 100 mM IPTG and 50µl of 2% X-gal was added to the L-agar. The Falcon tubes were held at 55°C until plating. 50 µl of an overnight culture of JM101 was added to the L-agar immediately before pouring, along with 100 µl of the tranfected cells. The cells were overlayed onto nutrient agar plates.

2.2.8 Propagation of M13 recombinants

Colourless plaques were picked into 5 ml of L-broth in universal bottles. 20 µl of an overnight culture of JM101 was added and the cells were grown at 37°C for six to eight hours. The cultures were spun down in an Eppendorf centrifuge for 5 minutes and then stored at 4°C with the clear supernatant over the cell pellet.

2.2.9 Amidase assay

Amidase was assayed in intact cells by the transferase assay of Brammar and Clarke (1964) using acetamide as substrate.

(a) Growth of bacteria

5 ml bacterial cultures were grown overnight at 37°C under inducing, non-inducing and repressing conditions. *E.coli* strains were grown in basal medium with nitrogen (Clarke & Laverack, 1983) containing glucose (0.5% w/v) or sodium succinate (0.5% w/v) as additional carbon source. This was supplemented with L-tryptophan or L-leucine at 0.003% (w/v) for growth of the auxotrophic strain JA221. *E.coli* strains were also grown in minimal media without nitrogen using glucose and succinate as carbon sources as described

above. L-tryptophan and L-leucine at concentrations of 0.01% (w/v) were added to the medium to provide a nitrogen limiting environment for growth. Lactamide (0.2% w/v) was used as an amide inducer and butyramide (0.2% w/v) was used as an amide analogue repressor of amidase synthesis. The media was supplemented with appropriate antibiotics.

P.aeruginosa strains were grown using the basal medium of Brammar and Clarke (1964) with ammonium sulphate (0.1% w/v) as nitrogen source and sodium succinate (1.0% w/v) or sodium lactate (1.0% w/v) as carbon sources. Inducing and repressing amides were added as for *E.coli.* The medium was supplemented with antibiotics as appropriate.

(b) **Hydroxamate transferase assay**

A solution of mixed substrates was freshly prepared using two volumes 0.1M Tris.HCl (pH 7.2), one volume 0.4M acetamide (freshly prepared) and one volume of 2M neutral hydroxylamine (10ml of 5M hydroxylamine hydrochloride neutralized to pH 7.2 with 10N NaOH and made up to 25 ml with distilled water) and kept on ice. 100 µl of a suitably diluted bacterial culture was added to 900 µl of mixed substrates in a 37°C water bath. After a ten minute incubation the reaction was stopped by the addition of 2 mls of ferric chloride/HCl (100ml 60% ferric chloride and 57 ml 12N HCl made up to one litre with distilled water). Reactions were vortexed to disperse bubbles of nitrogen and then read at 500 nm using a Unicam SP 600 spectrophotometer against a reagent blank. The absorbance was related to the amount of acetohydroxamate formed in the reaction by comparison with the standard curve (Brown, 1969). An absorbance of 1.0 at OD_{500} corresponds to 3.5 µmols/ml acetohydroxamate formed. Bacterial growth was measured at 670 nm for *P.aeruginosa* and 450 nm for *E.coli.* A standard curve was used to convert the readings to dry weights (Brown, 1969). An absorbance of 1.0 at OD_{670} corresponds to 0.56 mg dry weight/ml. A similar value is obtained for OD_{450} with *E.coli.*

(c) **Calculation of amidase specific activities**

The following equation was used in the calculation of amidase specific activities. The units of enzyme activity are μmol acethydroxamate formed/ min/mg bacteria:

$$\frac{A_{500} - (0.08 \times A_{670(450)}) \times 3.5}{(A_{670(450)} \times 0.56)}$$

A simple BBC basic computer program was written to process large numbers of amidase assays and to calculate specific activities.

Nucleic acid preparations/Manipulations

2.2.10 Ethanol Precipitation of DNA and RNA

DNA was routinely precipitated by the addition of one tenth volume of 3M potassium acetate followed by 2.5 volumes of ice cold ethanol. The mixture was vortexed then incubated on ice for ten minutes. The DNA was centrifuged for 15 minutes in an Eppendorf centrifuge and the supernatant discarded. 1ml of 70% ethanol was added to the pellet and the tube centrifuged again for 5 minutes. The supernatant was discarded and the pellet allowed to dry at room temperature for five minutes before resuspension in the appropriate buffer. RNA was precipitated in the same way except that 3M sodium acetate was used and three volumes of ethanol was added.

2.2.11 Phenol chloroform extraction of DNA

Protein was removed from DNA preparations by phenol/chloroform extraction. An equal volume of phenol/chloroform was added to the aqueous DNA sample and the mixture was vortexed for 30 seconds. The sample was then centrifuged in an Eppendorf centrifuge for two minutes and the upper aqueous phase was removed, avoiding any white interface.

2.2.12 Determination of nucleic acid concentrations

The concentration of nucleic acids was determined spectrophotometrically by measuring the absorbance at 260nm. An OD_{260} of 1 corresponds to a concentration of approximately 50 µg/ml for DNA, approximately 40 µg/ml for RNA and approximately 20 µg/ml for single-stranded oligonucleotides. The purity of DNA and RNA preparations was assessed by determining the ratio of absorbances at 260nm and 280nm Pure preparations of DNA and RNA have OD_{260}/OD_{280} values of 1.8 and 2.0 respectively. Samples with protein or phenol contamination had significantly lower OD_{260}/OD_{280} ratios.

2.2.13 Miniplasmid DNA preparation

The protocol used was a modification of the alkaline lysis method of Birnboim and Doly (1979) as described in Maniatis *et al* (1982).

1.5ml of a 5ml overnight culture was centrifuged for three minutes in an Eppendorf centrifuge. The supernatant was discarded and the pellet resuspended by vortexing in 100 µl of an ice cold solution of; 50mM glucose, 10mM EDTA, 25mM Tris.HCL pH 8.0, 4 mg/ml lysozyme (Solution I). Powdered lysozyme was added to the solution just before use. After five minutes at room temperature, 200 µl of a solution of 0.2N NaOH, 1% SDS (Solution II) was added and the tube contents mixed by inversion several times. The mixture was incubated on ice for five minutes. Then 150 µl of an ice cold solution of potassium acetate (60ml 5M potassium acetate, 11.5ml glacial acetic acid, 28.5 ml H_2O) (Solution III) was added and the tube vortexed in an inverted position for ten seconds. After five minutes on ice the tube was centrifuged for five minutes in an Eppendorf centrifuge and the clear supernatant transferred to a second tube. An equal volume of phenol/chloroform was added and the tube vortexed for thirty seconds. After centrifuging for 2 minutes the upper aqueous phase was transferred to a third tube. 2.5 volumes of ice cold ethanol was added and the tube incubated on ice for ten minutes. The tube was centrifuged for ten minutes and the supernatant discarded. The visible

pellet was washed once with 70% ethanol and then allowed to dry at room temperature. DNA pellets were resuspended in 40 µl TE buffer pH 8.0 (10mM Tris.HCL pH8.0, 1mM EDTA pH8.0) and RNase added to a final concentration of 20 µg/ml. The procedure described above was used to prepare plasmid DNA from both *P.aeruginosa* and *E.coli*.

2.2.14 Preparation of plasmid DNA for sequencing

Plasmid DNA was prepared for sequencing either by CsCl density centrifugation as described below, or by a modification of the miniplasmid method above, as described by Mierendorf and Pfeffer (1987).

2.2.15 Large scale isolation of plasmid DNA /CsCl density centrifugation

Large scale isolations of plasmid DNA were carried out using a modification of the alkaline lysis procedure described in section 2.2.13. The plasmid DNA isolated was purified by caesium chloride density centrifugation. Routinely 500 ml of culture was grown overnight and harvested by centrifugation in a Sorvall RC2-B centrifuge at 5000rpm, 4°C for 10 minutes. 20 ml of Solution I was added and the cells vortexed to ensure resuspension. The cells were incubated at room temperature for 5 minutes. 50 ml of Solution II was added and the cells stored on ice for 5 minutes. 50 ml of Solution III was added and the mixture inverted several times to mix. After five minutes on ice the mixture was spun in a Sorvall RC2-B centrifuge at 7000rpm, 4°C for 30 minutes. The supernatant was transferred to a fresh tube and 50 ml of 50% polyethylene glycol (PEG) 6000 was added. The mixture was then placed on ice for one hour. The plasmid DNA was precipitated by centrifugation for 30 minutes at 10,000rpm, 4°C and the pellet recovered and resuspended in 5 ml of TE buffer pH 8.0. The DNA solution was phenol/chloroform extracted twice. 1.1g CsCl was added per ml of solution and 200 µl of a 10 mg/ml solution of ethidium bromide. The solution was centrifuged in Beckman quick-seal polyallomer tubes at 50,000 rpm for twenty hours in a Beckman L-7 ultracentrifuge at 20°C. During the final 30 minutes of the ultracentrifugation the speed of the rotor was reduced

to 40,000 rpm to relax the gradient. The plasmid DNA band was removed from the tube by inserting a hypodermic needle through the side of the tube and retrieving the plasmid band. 4 ml of TE buffer was added to the DNA solution (approximately 1 ml) to avoid precipitation of the CsCl. The ethidium bromide was removed by extraction with an equal volume of CsCl saturated isopropanol several times. Finally the aqueous phase containing the plasmid DNA was ethanol precipitated and resuspended in 500 µl of TE buffer. The concentration of the DNA was determined spectrophotometrically as described previously. Typically this protocol yielded 0.5-1 mg of purified plasmid DNA depending on the copy number of the plasmid.

2.2.16 Isolation of chromosomal DNA

Chromosomal DNA was isolated by a modification of the method of Chater *et al* (1982). 10 ml of bacterial culture was grown overnight. The cells were pelleted in a Sorvall centrifuge at 5000 rpm, 4°C for ten minutes. The pellet was resuspended in 1 ml of Solution I (as above) and incubated at room temperature for five minutes. 100 µl of 10% SDS in distilled water was added and the solution inverted to mix. 500 µl of pronase (10mg/ml) was added and 4 ml of TE with RNAase at 20 µg/ml. The mixture was incubated at 37°C for one hour. A further 5 ml of TE buffer + RNAase was added and the mixture incubated for a further two hours at 37°C. 1 ml of 5M NaCl was added and the solution mixed. Then 15ml of ice cold ethanol was layered on top of the lysed cell solution and the chromosomal DNA spooled out on a Pasteur pipette. The viscous DNA was resuspended in 5 mls of TE buffer and an equal volume of phenol/chloroform was added. The solution was gently mixed for one hour to extract the protein. The mixture was then centrifuged and the upper aqueous phase removed, ethanol precipitated and resuspended in TE buffer. The concentration of the DNA was determined spectrophotometrically.

2.2.17 Isolation of single stranded M13 DNA

The method used was essentially as described in the BRL instruction

manual for M13 cloning/dideoxy sequencing. 1.2 ml of culture supernantant prepared as described previously was transferred to a 1.5 ml Eppendorf tube, 300 µl of 20% PEG6000/2.5M NaCl added and the tube inverted to mix. The solution was placed on ice for fifteen minutes. The mixture was centrifuged for 15 minutes and the supernatant discarded. The tube was then recentrifuged to bring down any residual supernatant and this was removed using a Gilson pipette. The phage pellet was resuspended in 100 µl of TE buffer and phenol/chloroform extracted twice. The upper aqueous phase was then ethanol precipitated and resuspended in 20 µl of TE buffer pH8.0. The concentration of the DNA was determined by agarose gel electrophoresis. To identify M13 recombinants with inserts in opposite orientations, the complementarity test was used (Perbal, 1988).

2.2.18 Isolation of RNA

All manipulations involving RNA used sterilised equipment. Solutions were made up with diethylpyrocarbonate (DEPC) treated water wherever possible. DEPC treated water was prepared as follows: DEPC 0.1% (w/v) was added to sterile distilled water and left overnight at room temperature. The solution was then autoclaved at 15 lb\in^2 for 20 minutes to remove the DEPC.

RNA was isolated using a modification of the method described by Deretic *et al* (1987). 500 ml of bacterial culture was grown to mid-exponential phase of growth ($OD_{450(670)}$= 0.5-0.8). The cells were pelleted by centrifugation in a Sorvall RC2-B centrifuge at 5000 rpm for 10 minutes at 4°C, washed with 5 ml of 50mM Tris.HCl pH 7.0 and recentrifuged. The cell pellets were then resuspended in 15mls of pre-warmed lysis buffer (50mM Tris.HCL pH7.0, 4% SDS, 6% phenol), vortexed and incubated at 56°C for five minutes. 5g of solid CsCl was added to the lysate and the solution vortexed to aid solublisation. The cell debris was removed by centrifugation in a Sorvall RC2-B centrifuge and the clear supernatant layered over a 2.5 ml cushion of 5.7M CsCl in a 13.2 ml Beckman Ultraclear open top tube (14x89mm). The sample was spun in a Beckman SW41Ti rotor in an L7 ultracentrifuge for 20 hours at 20° C. To remove the clear glass-like RNA pellet the solution above it was aspirated and

the tube cut just above the RNA pellet to produce a small "cup". 200 µl of sterile distilled DEPC treated water was added to the RNA pellet and the solution rapidly pipetted to aid dissolution. The RNA was then transferred to sterile Eppendorf tubes, ethanol precipitated and stored at -70°C.

2.2.19 Agarose gel electrophoresis of DNA

Agarose gels were run in a homemade horizontal flat bed apparatus. Gels were prepared and run in a Tris-borate buffer (90mM Tris, 90mM Boric acid, 2.5mM EDTA pH8.2). The percentage agarose used was determined by the size of DNA fragments to be separated according to Maniatis *et al* (1982). Samples were mixed with an appropriate volume of 5 x loading buffer (25% (w/v) Ficoll, 0.1M EDTA, 0.025% (w/v) bromophenol blue in Tris-borate buffer, pH8.2) and loaded into the sample wells. Gels were run at a field strength of 10 v/cm at room temperature. The DNA bands were visualised under UV irradiation from a 309 nm UV transilluminator (Ultra-Violet Products Inc.) after staining for thirty minutes in a solution of ethidium bromide (final concentration 1 µg/ml) and washing in distilled water. Gels were photographed using a Polaroid 4x5 type 55 positive-negative Land film with a 7x red filter on an MP4 Land camera. After developing, the negative was rinsed in a solution of 18% (w/v) anhydrous sodium sulphite followed by extensive washing with water. Photographs were also taken using Ilford HP-5 film and the same camera. These negatives were developed using Ilford microphen developer and fixer.

2.2.20 Isolation of DNA from agarose gels

Two methods were used to isolate DNA from agarose gels. The first method was essentially as described in Maniatis *et al* (1982). This method was superceded by the more convenient, faster method of M.Koenen (1989).

(a) Electroelution of DNA onto a dialysis membrane

The band to be excised was located under UV illumination and an incision was made in front of the leading edge of the band, about 2mm wider than the band on each side. A piece of Whatman 3MM paper backed with

dialysis tubing was inserted in the incision with the paper facing the DNA. The gel was returned to the tank and the DNA electrophoresed on to the paper at 200V for 5 minutes. A hole was pierced in the bottom of a 400 µl Eppendorf tube and this was placed inside a 1.5 ml Eppendorf tube. The paper with the DNA attached was placed inside the small Eppendorf tube and centrifuged for 30 seconds. The eluant in the lower tube was collected and 100 µl of elution buffer (0.2M NaCl, 50mM Tris pH7.6, 1mM EDTA and 1.0% SDS) was added to the paper, the tube was spun again and the eluant collected. This was repeated twice more and the fractions pooled. The DNA solution was phenol/chloroform extracted and ethanol precipitated and resuspended in TE buffer pH 8.0.

(b) **Rapid recovery of DNA from agarose gels**

This is a modification of the method described by Koenen (1989). The DNA was localised as above and the gel fragment containing the band was excised from the gel. A 2mm thick, siliconised sterile glass wool plug was placed in the bottom of a 400 µl Eppendorf tube with a hole pierced in the bottom. The gel slice was placed in this tube, which was then placed in a 1.5ml Eppendorf tube. The tubes were spun at 6000 rpm for ten minutes and the eluant collected. The eluant was phenol/chloroform extracted, ethanol precipitated and the DNA resuspended in TE buffer.

2.2.21 Polyacrylamide gel electrophoresis of DNA

(a) **Non-denaturing gels**

5% non-denaturing gels were used for DNA bandshift assays. Gels were prepared as described in Sambrook *et al* (1989), and run on a homemade vertical gel electrophoresis appuratus in 1x Tris Borate buffer (see above).

(b) **Denaturing gels**

Denaturing polyacrylamide gels were prepared exactly as described in the BRL M13 Cloning/Dideoxy Sequencing manual. 8% polyacrylamide/urea gels

were routinely used for DNA sequencing, primer extension analysis and S1 mapping.

2.2.22 Isolation of DNA from polyacrylamide gels.

DNA was isolated from polyacrylamide gels by a modification of the method of Sambrook *et al* (1989). The radioactive DNA band was located by autoradiography using radioactive ink as a marker to orientate the film with respect to the gel, the band was excised and placed in a 1.5 ml Eppendorf tube. The gel piece was crushed by rolling it around the side of the Eppendorf tube using a Gilson pipette tip and 200 µl of elution buffer (0.5M ammonium acetate, 1mM EDTA pH 8.0) was added. The tube was incubated overnight with gentle shaking at 37°C. A 2mm sterile siliconized glass plug was placed in the bottom of a 400 µl Eppendorf tube, with a hole pierced in the bottom. The crushed gel slice solution was placed in this tube, which was then placed inside a 1.5 ml Eppendorf tube. The tubes were spun in an Eppendorf centrifuge for 5 minutes at 12,000 rpm and the eluant recovered, phenol/chloroform extracted and ethanol precipitated.

2.2.23 Restriction enzyme digests

All restriction enzyme digests were carried out using the manufactures' recommended buffer. The volume of enzyme added to the reactions was never in excess of 10% of the total volume of the reaction, to avoid problems associated with high glycerol concentrations. Reactions were terminated by the addition of loading buffer if the digests were to be analysed by agarose gel electrophoresis or heat inactivation (68°C for ten minutes) if the DNA was to be used for subsequent digests or cloning.

2.2.24 DNA modification reactions

(a) Conversion of fragments with protruding 5' ends to blunt ends

Restriction fragments with protruding 5' ends were filled in using the DNA polymerizing activity of the Klenow fragment of *E.coli* DNA polymerase 1

(Maniatis *et al,* 1982). The restriction fragment was incubated at 22°C for 15-20 minutes in a final volume of 25 µl containing 2.5 µl 10x buffer (0.5M Tris.HCL pH7.2, 0.1M $MgSO_4$, 1mM DTT, 500 µg/ml BSA), 1 µl of a 2mM solution of all four dNTP's and five units of Klenow. The reaction was terminated by heating to 70°C for five minutes. The DNA was extracted with phenol/ chloroform, ethanol precipitated and resuspended in TE buffer.

(b) **Conversion of fragments with protruding 3' ends to blunt ends**

Restriction fragments with protruding 3' ends were made blunt ended using the 3' exonuclease activity of bacteriophage T4 DNA polymerase (Maniatis *et al,* 1982). The restriction fragment was incubated for five minutes at 37°C in a final volume of 20 µl with 2 µl 10x T4 polymerase buffer (0.33M Tris.acetate pH7.9, 0.66M potassium acetate, 0.1M magnesium acetate, 5mM DTT, 1mg/ml BSA), 1 µl of a 2mM solution of all four dNTP's and 1 µl T4 DNA polymerase (10 units). The reaction was terminated by the addition of 1 µl of 0.5M EDTA. The DNA was extracted with phenol/chloroform, ethanol precipitated and resupended in TE buffer.

2.2.25 Dephosphorylation of DNA

Dephosphorylation of DNA prior to 5' end labelling or, of vector DNA to prevent recircularisation during ligation was achieved using calf intestinal alkaline phosphatase (CIP). DNA was incubated in 1x CIP buffer (50mM Tris.Cl pH9.0, 1mM $MgCl_2$, 0.1mM $ZnCl_2$, 1mM spermidine) with 1-5 units of CIP at 37°C for one hour. The reaction was terminated by adding SDS to a final concentration of 0.5% and heating the reaction mixture to 68°C for fifteen minutes. The reaction mixture was subsequently phenol/chloroform extracted twice and the DNA ethanol precipitated.

2.2.26 Ligation of DNA

Cohesive and blunt end DNA ligations were carried out under identical conditions. Ligations were typically carried out in a volume of 10 µl with 1 µl

of 10x ligation buffer (0.66M Tris.HCL pH7.6, 50mM $MgCl_2$, 50mM DTT 10mM ATP), 1 µl T4 DNA ligase (10 units) at 16°C overnight.

2.2.27 ^{32}P labelling of DNA

(a) End labelling of DNA fragments and oligonucleotides

5'-end labelling of DNA was carried out using T4 polynucelotide kinase (PNK) and ^{32}P-γ-ATP. To 5'-end label a restriction fragment or linearised plasmid the overhanging 5' phosphate was removed by treatment with CIP as described in section 2.2.25. Oligonucleotides to be 5'-labelled did not require this step as they were made without a 5'-phosphate group. 1 µg of plasmid or 100 ng of oligonucleotide was incubated at 37°C for one hour in a reaction containing 50mM Tris.HCL pH7.6, 10mM $MgCl_2$, 1mM DTT, 1mM EDTA, 1mM spermidine, 100 µCi of ^{32}P-γ-ATP (3000 Ci/mmol) and 10 units of T4 PNK. The reaction was terminated by heating to 65°C for 15 minutes. Unincorporated ATP was removed from the labelled DNA using a NAP5 column (Pharmacia) following the manufacturers instructions. The column was equilibrated with 0.5 ml TE buffer pH8.0 and the DNA/oligonucleotide eluted in 1ml TE buffer pH8.0. Oligonucleotides and DNA fragments prepared in this way were then used directly in subsequent manipulations and had a specific activity of between 10^8-10^9 cpm/µg DNA as determined by liquid scintillation counting of the DNA.

(b) Random priming oligonucleotide labelling

Uniformly labelled DNA probes for Northern and Southern analysis were prepared using the Boehringer Mannheim random primed DNA labelling kit and ^{32}P-α-dCTP (3000Ci/mmol). Routinely 25-100 ng of DNA was labelled following the manufacturers instructions. Unincorporated nucleotides were removed from the labelled probe by diluting the reaction mix five fold with TE buffer pH 8.0 and spinning it through a G-50 Sephadex column (pre-equilibrated in TE buffer pH8.0) at 2000 rpm for five minutes in a bench top cen-

trifuge as described in Maniatis *et al*, (1982). The purified probe was denatured immediately prior to use by the addition of sodium hydroxide to a final concentration of 0.1 N and incubation at 37°C for five minutes.

2.2.28 Northern analysis

RNA samples were analysed on 1% formaldehyde gels prepared by the method of Perbal (1988). Prior to electrophoresis the RNA sample (10-30 µg) was ethanol precipitated and resuspended in 5 µl of DEPC treated water. 3.5 µl of 40% formaldehyde solution was added with 2 µl of 5x running buffer (200mM MOPS, 50mM sodium acetate pH7.0, 10mM EDTA) and 10 µl of deionised formamide. The solution was incubated at 55°C to denature the RNA and 5 µl of agarose gel loading buffer was added to the sample prior to electrophoresis. Formaldehyde gels were electrophoresed in 1x running buffer (see above) at 35 mA for 4-6 hours. Following electrophoresis the RNA was visualised by UV illumination and photographed. A BRL RNA ladder was used as a molecular weight marker. The RNA was then transferred directly from the gel to Biodyne A membrane by the standard capillary blot technique (Maniatis *et al*, 1982) following the manufacturers instructions. Following transfer the RNA was immobilised on the filter by baking in a vacuum oven for two hours at 80°C.

Filters were prehybridised and hybridised in a solution containing 5x Denhardt's solution (100x Dernhardt's contains: 2% (w/v) Ficoll (400,000 Mwt), 2% (w/v) Polyvinylpyrrolidone (360,000 Mwt), 2% (w/v) BSA), 5x SSC (20x SSC contains: 3M NaCl, 0.3M Sodium citrate pH 7.0), 50mM sodium phosphate pH 6.5, 0.1% SDS, 250 µg/ml denatured Salmon sperm DNA, 50% (v/v) formamide. Pre-hybridisation was for one hour and hybridisation was for 14-16 hours. Prior to hybridisation the denatured radioactively labelled probe was added to the hybridisation solution. Pre-hybridisation and hybridisation reactions were carried out at 50°C. Following hybridisation the filters were washed twice in 2x SSC, 0.1% SDS for 15 minutes, and twice in 0.1x SSC, 0.1% SDS at 65°C prior to

autoradiography at -70°C with an intensifying screen.

2.2.29 Southern analysis

DNA samples were separated on 0.7% agarose gels as described above. Following electrophoresis the gel was photographed with UV illumination and the DNA denatured as follows: the gel was soaked in 0.25M HCl for 20 minutes for depurination, rinsed for five minutes in distilled water then soaked for 20 minutes in 0.4M NaOH. The gel was then blotted using an alkali blot procedure with Hybond N+ membrane as described in the manufacturers instructions. Blotting was performed for 14-16 hours followed by two rinses of the filter in 2x SSC, 0.1% SDS. Filters were pre-hybridised and hybridised in a solution containing 6x SSC, 5x Denhardt's, 0.5% SDS and 100 μg/ml denatured Salmon sperm DNA. Pre-hybridisation was for 4-5 hours and hybridisation in the presence of a radioactively labelled probe was for 14-16 hours at 65°C. Filters were subsequently washed twice in 2x SSC for 15 minutes at room temperature, followed by two washes at 65°C in 0.1x SSC. Filters were then subjected to autoradiography at -70°C with an intensifying screen.

2.2.30 Primer extension analysis

Primer extension analysis was carried out using end-labelled oligonucleotides prepared as described in section 2.2.27. RNA was hybridised to the oligonucleotide by the method of Geliebter (1987): Approximately 300 pg of oligonucleotide (1-5 x 10^5 cpm) was co-precipitated with 20-40 μg of RNA. The pellet was resuspended in 25 μl of sterile distilled water and 25 μl of 2x hybridisation buffer was added (0.5M KCl, 20mM Tris.HCL pH 8.3). The solution was heated to 85°C for five minutes and then incubated at a temperature 5°C below the T_d of the oligonucleotide (T_d (^{0}C) = ((4 x G+C content)+ (2 x A+T content))) for one hour followed by ethanol precipitation.

The hybridised RNA/DNA pellet was resuspended in 3 μl H_2O and the

following added: 10 µl 2x reaction buffer (80mM Tris.HCL pH8.3, 80mM KCl, 16mM $MgCl_2$, 5 µl of a 2mM solution of all four dNTP's, 1 µl (25 units) placental RNase inhibitor (Promega), 1 µl avian myeloblastosis virus reverse transcriptase (AMV-RT, Anglian Biotechnology). The reaction mixture was incubated for one hour at 50°C to minimise RNA secondary structure formation. The extension products were recovered by ethanol precipitation and resuspended in sequencing gel loading buffer (formamide 10ml, xylene cyanol FF 10mg, bromophenol blue 10mg, 0.5M EDTA pH8.0 200µl) and subsequently resolved on 8% polyacrylamide/urea sequencing gels. Sequencing reactions were run simultaneously to provide suitable size markers. Following electrophoresis the gel was transferred to Whatman 3MM paper, covered with Saran wrap, dried down using a Biorad gel dryer and subjected to autoradiography at -70°C with an intensifying screen.

2.2.31 S1 nuclease analysis

S1 nuclease mapping (Burke, 1984) was used to identify the 3' end of the *amiE* transcript, and the 5' end of the *amiCR* transcript. Single stranded probes which were either end-labelled or continuosly labelled were created from M13 DNA templates, isolated on denaturing polyacrylamide gels and hybridised to RNA prior to S1 nuclease digestion. For generation of end-labelled probes for mapping of the 5' ends of transcripts, 10 ng of end-labelled oligonucleotide complementary to a region 100-300 bp from the transcription start, was annealed to 1 µg of a single stranded recombinant containing the appropriate sequence, in a solution containing 10mM Tris.HCL pH 7.5, 10mM $MgCl_2$, by heating to 70°C for five minutes and allowing to cool slowly to 37°C. The complementary strand of the M13 recombinant was then formed in the presence of five units of Klenow enzyme and 125 µM of all four dNTP's. After terminating the reaction by heating to 65°C for 15 minutes the solution was adjusted to the appropriate salt concentration and the newly synthesised

double stranded DNA was cleaved with an appropriate restriction enzyme by incubation for one hour at 37°C. The reaction was terminated by the addition of EDTA to a final concentration of 15mM and ethanol precipitation. The DNA was collected by centrifugation, resuspended in 10 µl sequencing loading buffer, and denatured by heating to 90°C for three minutes. The denatured sample was loaded onto an 8% polyacrlyamide/urea gel for electrophoresis at 70 W until the loading dyes indicated sufficient separation. One of the glass plates was removed and the gel covered with Saran wrap before exposure to an X-ray film with an intensifying screen for 15 minutes. The DNA band was then recovered from the gel as described in section 2.2.22.

Continuously labelled probes were generated by the method of Burke (1984) using α-^{32}P-dCTP (3000Ci/mmol) with either M13 universal primer or oligonucleotide C. The restriction digest was terminated as above and the probe ethanol precipitated. The probe was resuspended in 10 µl of sequencing loading buffer, then denatured, electrophoresed and purified as above. For S1 mapping using end-labelled probes or continuously labelled probes, 2-5 x 10^4 cpm of purified probe was used per reaction. The hybridisation of the probe to the RNA and digestion with S1 nuclease was as described by Burke (1984). S1 nuclease digestion was terminated by the addition of 4 µl of 0.25M EDTA and 10 µg tRNA followed by ethanol precipitation. The S1 resistant products were resuspended in sequencing gel loading buffer, denatured by heating to 90°C for three minutes then analysed on an 8% polyacrylamide/urea sequencing gel. The gel was subsequently dried down and subjected to autoradiography at -70°C with an intensifying screen.

2.2.32 DNA sequencing

Sequencing reactions for both plasmid DNA and single stranded M13 were carried out using Pharmacia deaza-GTP sequencing premixes, Klenow enzyme, ^{35}S-dATP(1000-1500Ci/mmol) and universal sequencing primer. Sequencing reactions were carried out according to the manufacturers instructions except that reactions were carried out at 37°C. Samples were denatured

in sequencing gel loading buffer for three minutes at 90°C and immediately loaded onto 8% polyacrylamide/urea gels and electrophoresed at 70W. Double fine sharktooth combs (BRL) were used to load the samples. Three loadings of each sequencing reaction were done on the same gel with the following loading times: first loading at 0 hours, second loading at three hours, third loading at five hours. The electrophoresis was stopped when the bromophenol blue dye from the third loading had reached the bottom of the gel. Following electrophoresis the gel was transferred to Whatman 3MM paper, covered with Saran wrap and dried down using a Biorad gel dryer. Autoradiography was performed at room temperature without an intensifying screen. A example of a sequencing gel is shown in Figure 2.1

2.2.33 Protein Analysis

(a) **Preparation of cell free extracts**

Bacteria were routinely grown in 50ml of nutrient medium at 37°C with appropriate antibiotics to an $OD_{450(670)}$ of 0.5. IPTG was added to appropriate cultures (final concentrations: 1mM for *E.coli* and 5mM for *P.aeruginosa*) and incubation was continued for a further four hours. Cultures were chilled on ice for ten minutes and the cells harvested by centrifugation in a Sorvall RC2-B centrifuge at 5000rpm, 4°C, for ten minutes. The cell pellet was resuspended in 1/50 original volume of cell lysis buffer (20mM Tris.HCl pH8.0, 1mM DTT, 0.5mM PMSF, 5mM EDTA) and disrupted by sonication on ice using an MSE Soniprep 150. For sonication ten, 15 second bursts at an amplitude of 14 were applied to the sample with a cooling interval of 15 seconds between each burst. Cell debris was removed from the cell free extracts by centrifugation in an Eppendorf centrifuge at 12000rpm for five minutes. Cell free extracts were stored at -70°C after the addition of 15%(v/v) sterile glycerol.

(b) **SDS-polyacrylamide gel electrophoresis**

For the analysis of cell free extracts and to monitor the purification of

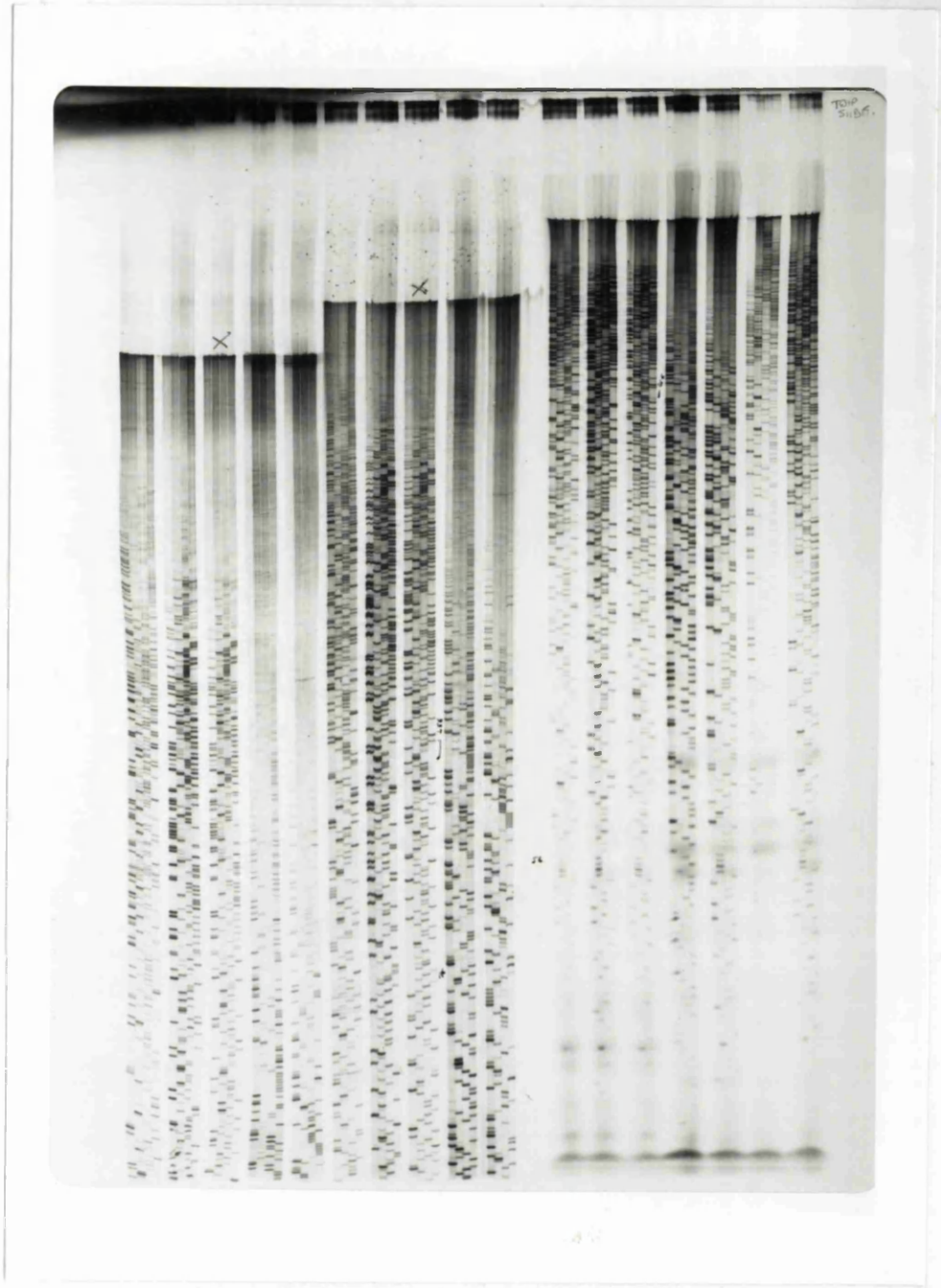


Figure 2.1. Typical sequencing gel obtained using the protocol described in Section 2.2.32.

AmiC, SDS-polyacylamide gels were used as described by Laemmli (1970). A homemade vertical gel electrophoresis tank was used to run gels. The concentration of acrylamide in the gels was determined by the size of proteins to be resolved. Generally gels between 8-12.5% acrylamide were used.

For a 12% resolving gel, 12ml of a 30% (w/v) stock acrylamide solution (28.2g acrylamide, 0.8g N-N-methylene bisacrylamide in 100ml water, filtered and stored in the dark at 4°C) was added to 7.5ml of 1.5M Tris.HCL pH8.8, 0.4% (w/v) SDS buffer and 1.5ml of ammonium persulfate (1.5%, w/v) and made up to 30ml with water. 23µl of TEMED was added to the solution and the gel poured. Polymerisation took approximately 30 minutes.

For a 3% stacking gel, 1ml of 30% (w/v) acrylamide stock solution was added to 2.5ml of 0.5M Tris.HCL pH6.8, 0.4% (w/v) SDS buffer and 0.5ml of ammonium persulfate (1.5% w/v) and made up to 10ml with water. 10µl of TEMED was added to the solution and the stacking gel poured on top of the resolving gel. The stacking gel was left for 45 minutes to polymerise.

Samples were diluted 1:1 (v/v) with loading buffer (0.0625M Tris.HCL pH6.8, 2% (w/v) SDS, 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol, 0.001% (w/v) bromophenol blue) and heated to 100°C for five minutes prior to loading. Protein molecular weight markers (Sigma) were run concurrently with test samples. Electrophoresis was carried out in a Tris-Glycine-SDS buffer (0.025M Tris.HCL pH8.3, 0.192M glycine, 0.1% SDS) at 35mA constant current for approximately four hours, until the dyefront had reached the bottom of the resolving gel. Following electrophoresis the gel was stained with pre-prepared Coumassie Brilliant Blue R stain (Sigma) for 14-16 hours and then destained with destain solution (50% methanol (v/v), 10% acetic acid (v/v) in water) for approximately 3-4 hours.

(c) **Gel filtration of crude extracts containing AmiC**

Gel filtration of crude cell extracts was carried out using an FPLC Sy-

stem III machine (Pharmacia). 200µl (approximately 10mg protein) of crude extract (prepared as above) was applied to a Superose HR 10/30 gel filtration column prewashed with elution buffer (EB) (20mM Tris.HCL pH8.0, 1mM DTT, 1mM EDTA) and eluted with EB at a flow rate of 0.3ml/min. Fractions were collected with a Frac-100 fraction collector (Pharmacia) and stored at 4°C for subsequent analysis

(d) **Ion exchange chromotography of AmiC**

Ion exchange chromatography was carried out using the FPLC system described above. Fractions from the gel filtration column were loaded individually onto a MonoQHR 5/5 ion exchange column prewashed with EB. The column was eluted with a linear gradient of 0-0.6M NaCl at a flow rate of 1.0 ml/min. Fractions were collected as above and stored at 4°C for subsequent analysis. Fractions subsequently shown to contain purified AmiC were stored at -70°C in EB with 15% sterile glycerol (v/v).

2.2.34 Gel retardation assays

Gel retardation assays were carried out by a modification of the method of Pfeifer *et al* (1987). Standard reactions were carried out in a total volume of 20 µl as follows: 1-2 ng of end-labelled target DNA (2-5x10^5 cpm) was incubated with 10 µl of 2x binding buffer (8mM Tris-HCL (pH8.0), 80mM NaCl, 8mM $MgCl_2$, 10% glycerol), 15-150ng purified AmiC (or 1 µl crude extract containing amiC, prepared as described above), 1 µg sheared salmon sperm DNA (non-specific competitor), at 24°C for thirty minutes. Following incubation the samples were loaded directly onto a 5% non-denaturing polyacrylamide gel, without adding loading buffer. Samples were electrophoresed at a constant current of 35mA until marker dye (bromophenol blue) had reached the bottom of the gel. Gels were subsequently transferred to Whatman 3MM paper, covered with Saran wrap and dried down. Gels were then subject to autoradiography at -70°C with an intensifying screen.

2.2.35 Filter binding assays

Filter binding assays were carried out using a modification of the method of Riggs *et al,* (1969). End-labelled pAS20 was prepared by cleavage at the unique *Hin*dIII target, followed by dephosphorylation and end-labelling as described above. Filter binding reactions were carried out in a total volume of 200 µl as follows: 1-2ng end-labelled pAS20 ($3-5x10^5$ cpm) was added to 6 µg of sheared Salmon sperm DNA, 0.75-4.5µg purified AmiC, 100 µl of 2x binding buffer (0.02M magnesium acetate, 0.01M KCl, 10^{-4}M EDTA, 10^{-4}M DTT, 5% DMSO, 0.01M Tris-HCL pH7.4, 50µg BSA). Samples were incubated at 24°C for thirty minutes and then filtered as follows: Nitrocellulose filters (Schleicher & Schuell) were presoaked for fifteen minutes in filter buffer (Filter buffer = binding buffer without BSA or DTT) and then mounted in a slot blot appuratus (Schleicher & Schuell), reactions mixtures were split into two aliquots and filtered seperately. Filtering was carried out under vacuum with a flow rate of approximately 50 µl/minute. Following filtration of the reaction mixture, 100 µl of FB buffer was filtered through each slot to wash off any non-specifically bound DNA. Following the wash the nitrocellulose filter was removed and cut up and the radioactivity retained on each slot determined by liquid scintillation counting. Values obtained from the same reaction mixture were averaged.

2.2.36 Protein kinase assay gels

The protein kinase activity of purified AmiC was monitored by incubation of purified AmiC with crude cell extracts from *P.aeruginosa* in the presence of ^{32}P-γ-ATP, followed by SDS polyacrylamide gel electrophoresis of the samples and autoradiography of the dried down gel. Labelling reactions were carried out as follows: 7 µl of a crude extract prepared as described above was added to 10µl purified AmiC in elution buffer (approximately 1.5µg), 1µl ^{32}P-γ-ATP (=1µCi ; specific activity = 3000Ci/mmol), 12 µl H_2O. Where appropriate 1µl of 10% (w/v) DL-lactamide was also added to the reaction mixture. Samples were incubated at 37°C for 30 minutes. To terminate the reaction and denature the proteins, an equal volume of sample buffer was added to the

reaction mixtures and the samples incubated for a further thirty minutes at 37°C. Following denaturation the samples were loaded onto a 12% SDS-PAGE gel and electrophoresed as in section 2.2.33, along with molecular weight markers (Sigma). Following electrophoresis the lane containing the molecular weight markers was excised from the gel and stained and destained as described in section 2.2.33. The remainder of the gel was transferred to Whatman 3MM paper, covered with Saran wrap and dried down using a Biorad gel dryer. The dried down gel was subject to autoradiography at -70°C with an intensifying screen.

2.2.37 N-terminal amino acid sequencing

The FPLC purified AmiC was concentrated and desalted by HPLC using a Brownlee Aquapore RP300 (2.1mm id x 60mm - Anachem, Bedford) with a solvent gradient of 5-65% acetonitrile in water at 0.13 ml/min. The acetonitrile contained 0.04% and the water 0.06% trifluoroacetic acid respectively and absorption was monitored at 214nm. Apart from the salt flow-through a single large peak with a retention time of 21.8 minutes was seen. The peak was collected and used for N-terminal amino acid sequence analysis. Approximately 80pmols (5 μg) of HPLC purified AmiC was subject to N-terminal amino acid sequence analysis. The first 20 amino acids were sequenced by automated Edman degredation using an Applied Biosystems 470A Gas Phase Sequencer with on line detection of amino acid phenylthiodantoin derivatives using 120A HPLC (Applied Biosystems, Warrington U.K.). The AmiC polypeptide gave a single amino acid sequence with initial yield of 70% and repetitive yields of 91%.

CHAPTER THREE - ATTEMPTS TO IDENTIFY THE *amiR* PROMOTER AND THE DISCOVERY AND SEQUENCING OF *amiC*

3.1 Introduction

At the time this work started the wild type amidase genes from PAC1 and the genes from the constitutive mutant PAC433 had been cloned. These fragments had been subcloned into pBR322 using *Hin*dIII and *Sal*I to generate plasmids pAS20 (PAC1 genes) and pJB950 (PAC433 genes). The location and direction of transcription of *amiE* and *amiR* had been determined and an extensive restriction map of the cloned *Pseudomonas* DNA had been generated. This information is summarised in Figure 3.1. The first experimental goal was to try and identify the *amiR* promoter.

The location of the *amiR* gene was initially determined using *in vitro* constructed deletions in pJB950 (Cousens *et al*, 1987). This study attempted to define the *amiR* promoter region using constructs with unique 3' ends (*Sal*I) and various lengths of upstream sequence. The result of this study placed the *amiR* promoter, between the *Kpn*I (2356) and *Cla*I(3266) targets (see Figure 3.1). The available DNA sequence of part of this region (*Xho*I (3000) to *Cla*I (3266) was of no assistance in promoter identification (N.Lowe and R.E. Drew, unpublished information). The *Kpn*I-*Cla*I fragment shown to contain a promoter was approximately 950bp long and the initial aim of the following experiments was to localise this promoter more precisely. The experiments used the recently cloned wild type genes in plasmid pAS20 and the genes from the constitutive mutant PAC433 in plasmid pJB950.

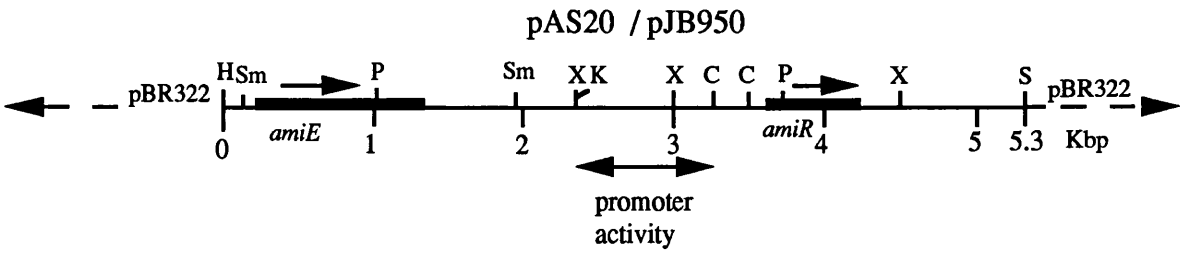


Figure 3.1. Restriction map of the *Hin*dIII-*Sal*I fragments of plasmids pJB950 (PAC433 genes) and pAS20 (PAC1 genes), which were subcloned into the *Hin*dIII-*Sal*I targets of pBR322. The direction of transcription of *amiE* and *amiR* are shown by the arrows. Abbreviations for restriction enzyme targets are as follows: H= *Hin*dIII, P= *Pvu*II, X= *Xho*I, K= *Kpn*I, C= *Cla*I, S= *Sal* I, Sm = *Sma* I The *Sma* I target upstream of the *amiE* gene comprises two sites spaced by 10bp which form part of the transcription terminator which regulates amidase expression.

3.2 *In vitro* deletions/rearrangements to identify the *amiR* promoter

To locate the *amiR* promoter, the wild type and constitutive cloned genes in pAS20 and pJB950 were rearranged, and regions deleted. The newly constructed plasmids were characterised and assayed for amidase activity in *E.coli*.

3.2.1 Construction of plasmids pSW1, pSW2 and pSW5

Plasmid pJB950 was digested with *Xho*I and *Sal*I and the mixture of fragments ligated and used to transform *E.coli* JA221 to ampicillin resistance. Plasmid DNA was isolated from transformants and three new recombinant plasmids were identified by restriction mapping using *Hin*dIII, *Xho*I, *Kpn*I, *Cla*I and *Pvu*II and are shown in Figure 3.2. pSW1 is missing the 800bp *Xho*I-*Sal*I fragment. It has the 1.5kb *Xho*I *amiR* fragment in the normal orientation and the 658bp *Xho*I fragment in the reverse orientation. Plasmid pSW2 has the 800bp *Xho*I-*Sal*I and the 658bp *Xho*I fragments deleted and the 1.5kb *Xho*I *amiR* fragment is in the reverse orientation. Plasmid pSW5 has the 800bp *Xho*I-*Sal*I and the 658bp *Xho*I fragments deleted with the 1.5kb *Xho*I *amiR* fragment in the normal orientation.

3.2.2 Amidase expression from pJB950, pSW1, pSW2 and pSW5 in *E.coli*

Wild type *E.coli* does not possess amidase activity although the controls may give a trace colour reaction in the transferase assay due to non-specific esterase activity (Lipmann & Tuttle, 1945). Expression of amidase from pJB950, pSW1, pSW2 and pSW5 was measured under non-inducing, inducing and repressing conditions using lactamide as inducer and butyramide as an amide analogue repressor and the results are shown in Table 3.2. It has been shown previously that *Bal*31 generated deletions encompassing the 800bp *Xho*I-*Sal*I fragment have no significant effect on amidase expression, thus the deletion of this fragment in these constructs is not considered to be the reason for changes in amidase expression.

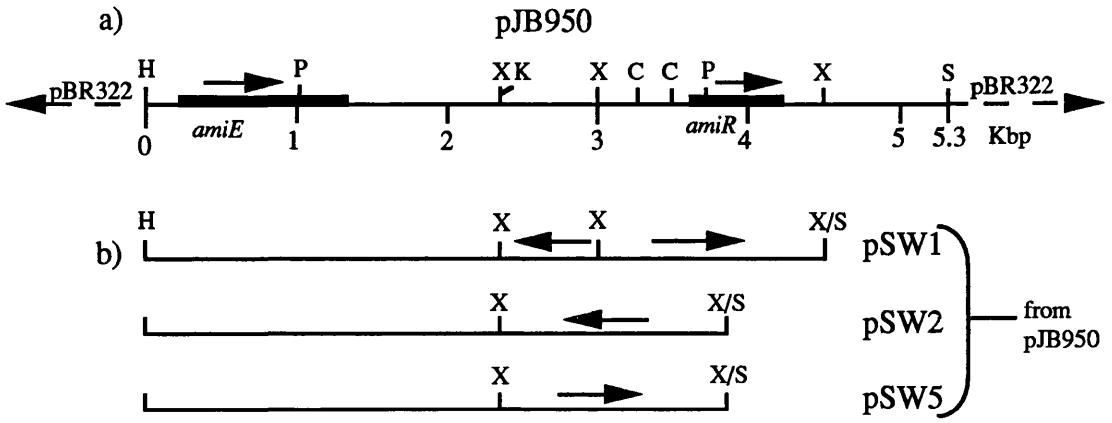


Figure 3.2 a) Restriction map of the *Hin*dIII-*Sal*I PAC433 DNA fragment inserted in plasmid pJB950 and pAS20, which were subcloned into the *Hin*dIII-*Sal*I targets of pBR322. The location and direction of transcription of *amiE* and *amiR* are shown by the arrows. Abbreviations for restriction enzyme targets are as follows: H= *Hin*dIII, P= *Pvu*II, X= *Xho*I, K= *Kpn*I, C= *Cla*I, S= *Sal* I

b) Restriction maps of plasmids pSW1,2 and 5. These plasmids are all derivatives of plasmid pJB950. The arrows indicate the orientation of fragments within these plasmids. X/S corresponds to the hybrid site generated by ligation of an *Xho*I target and a *Sal*I target.

TABLE 3.1. Amidase activities[a] from *E.coli* JA221 carrying plasmids pJB950 and reconstructed derivatives.

Plasmid	Growth Conditions					
	Glucose	Glucose/ Lactamide	Succinate	Succinate/ Lactamide	Succinate/ Butyramide	Mean
pJB950	3.8	4.0	3.2	3.8	3.5	3.7
pSW1	0.4	0.3	0.3	0.3	0.3	0.3
pSW2	0.5	0.3	0.2	0.3	0.3	0.3
pSW5	7.0	7.8	9.9	7.8	10.9	8.7

a) Amidase activity was measured as μmoles acetohydroxamate produced per minute per mg bacteria. Values shown are the mean of duplicate assays carried out on at least three separate occasions.

Plasmid pJB950 gives low constitutive expression under all growth conditions (Cousens *et al*, 1987). Plasmid pSW1 with the inversion of the 658bp *Xho*I fragment gives a ten fold decrease in amidase expression, suggesting this fragment of DNA contains a promoter for *amiR* and when inverted the promoter is non-functional. Plasmid pSW2 with the 1.5kb *Xho*I *amiR* fragment in the opposite orientation and the 658bp *Xho*I fragment deleted also shows a ten fold decrease in amidase expression compared to pJB950. In this construct the *amiR* gene still carries 650bp of upstream sequence and yet gives negligable amidase expression, suggesting that the *amiR* promoter does not lie within the 1.5kb *Xho*I fragment. Plasmid pSW5 has a deletion of the 658bp *Xho*I fragment and gives high levels of amidase expression under all growth conditions. *Bal*31 generated deletions have previously been made in this region and these also showed an increase in amidase expression (Cousens *et al*, 1987). These findings suggest that *amiR* can be expressed from a promoter upstream of the 658bp *Xho*I fragment.

3.2.3 Construction of plasmids pSW3 and pSW4

Plasmid pAS20 was digested with *Xho*I and *Sal*I and the mixture of fragments ligated and used to transform *E.coli* JA221 to ampicillin resistance. Plasmid DNA was isolated from transformants and plasmids pSW3 and pSW4 identified by restriction mapping with *Hin*dIII, *Xho*I, *Kpn*I, *Cla*I and *Pvu*II . Restriction maps of these plasmids are shown in Figure 3.3. Plasmid pSW3 has the 800bp *Xho*I-*Sal*I fragment and the 658bp *Xho*I fragments deleted and the 1.5kb *Xho*I *amiR* fragment in the normal orientation. Plasmid pSW4 is the same as pSW3 except that the 1.5kb *Xho*I *amiR* fragment is in the reverse orientation.

3.2.4 Amidase expression from pAS20, pSW3 and pSW4 in *E.coli*

Amidase expression was measured in *E.coli* JA221 under the growth conditions described above and the results are shown in Table 3.3. Plasmid pAS20 shows no amidase activity except when grown under inducing conditions in glucose. This could be due to poor expression of the *amiR* gene in this heterologous host. However, pAS20, containing the wild type genes does not have the point mutation in the ribosome binding site upstream of *amiE*, found in pJB950 which leads to a four fold increase in amidase expression from this plasmid (Drew and Lowe, 1989). Plasmid pSW3 shows constitutive amidase expression under all growth conditions, and this expression is at a higher level than that seen with pAS20 grown under inducing conditions. This result suggest that i) deletion of the 658bp *Xho*I fragment allows expression of *amiR* from an upstream promoter (as in pSW5) ii) because expression is now constitutive and the original plasmid contained wild type inducible genes, the 658bp *Xho*I fragment contains either a *cis* acting sequence regulating *amiR* expression or this region of DNA encodes for a gene regulating amidase expression.

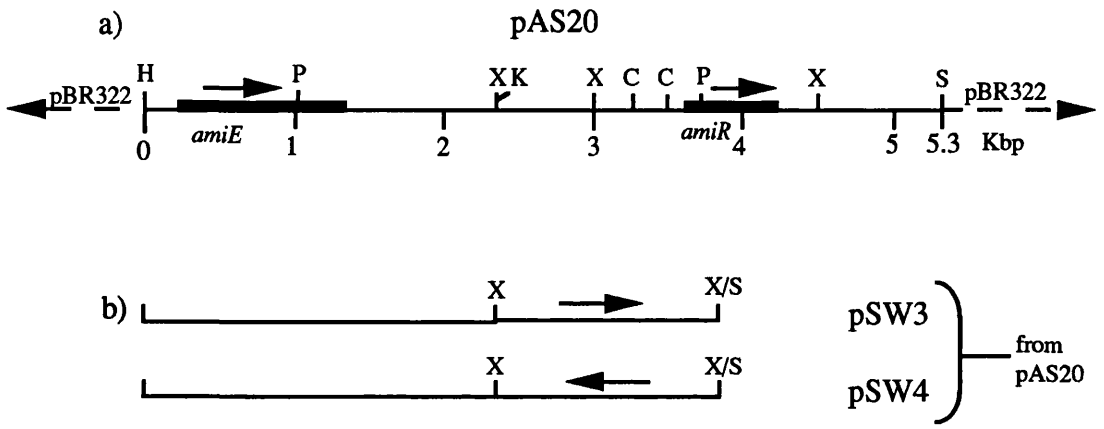


Figure 3.3 a) Restriction map of the *Hin*dIII-*Sal*I PAC1 DNA fragment in plasmid pAS20. The direction of transcription of *amiE* and *amiR* are shown by the arrows. Abbreviations for restriction enzyme targets are as follows: H= *Hin*dIII, P= *Pvu*II, X= *Xho*I, K= *Kpn*I, C= *Cla*I, S= *Sal* I

b) Restriction maps of plasmids pSW3 and pSW4. These plasmids are derived from plasmid pAS20. The arrows indicate the orientation of fragments within these plasmids. X/S corresponds to the hybrid site generated by ligation of an *Xho* I target and a *Sal* I target.

TABLE 3.2. Amidase activities[a] from *E.coli* JA221 carrying plasmid pAS20 and reconstructed derivatives.

Plasmid	Growth Conditions					
	Glucose	Glucose/ Lactamide	Succinate	Succinate/ Lactamide	Succinate/ Butyramide	Mean
pAS20	0.0	0.1	0.0	0.0	0.0	0.0
pSW3	2.1	1.6	1.6	1.6	1.6	1.7
pSW4	0.1	0.1	0.0	0.0	0.0	0.0

a) Amidase activity was measured as μmoles acetohydroxamate produced per minute per mg bacteria. Values shown are the mean of duplicate assays carried out on at least three separate occasions.

This finding also suggests that the induction process does not involve a direct interaction between AmiR and inducer since plasmid pSW3 has a wild type *amiR* gene which is now shown to function in the presence and absence of inducer. The implication of this result is that either AmiR activity, or *amiR* expression is regulated by some factor present in the 658bp *Xho*I fragment. Plasmid pSW4 is structurally similar to plasmid pSW2 described above and shows negligible amidase expression, suggesting the *amiR* promoter does not lie within the 1.5kb *Xho*I *amiR* fragment.

3.3 Evidence that *amiR* functions independently of inducer

To confirm that the wild type *amiR* gene functions independently of inducer a transcomplementation system was set up in *E.coli* JA221 with *amiR* expressed from a constitutive vector promoter, and the *amiE* gene expressed from its normal promoter on a separate plasmid.

3.3.1 Construction of plasmid pSW35

Plasmid pSW35 was constructed as follows: the wild type *amiR* gene was isolated from pAS20 on the 1.5kb *Xho*I fragment and ligated to the broad host range vector pKT231 which had been linearised with *Xho*I. The *Xho*I target in pKT231 lies within the kanamycin resistance gene, thus plasmids containing an insertion at this site are Km^s. The ligation mixture was used to transform *E.coli* JA221 to streptomycin resistance. Potential recombinant plasmids were identified as streptomycin resistant, kanamycin sensitive transformants. pSW35 was identified as carrying the *amiR* gene fragment in the correct orientation for expression from the vector neomycin phosphotransferase II promoter by restriction mapping of plasmid DNA isolated from these transformants. The structure of plasmid pSW35 and pDC5 are shown in Figure 3.4. Plasmid pSW35 expresses *amiR* constitutively from the vector neomycin phosphotransferase II promoter as shown previously for the isogenic plasmid pDC35 (Cousens *et al*, 1987).

3.3.2 Transcomplementation with plasmids pSW35 and pDC5 in *E.coli*

Plasmid pSW35 was used in a transcomplementation system with pDC5 in *E.coli*. pDC5 is a deletion derivative of pJB950 which just carries the 2.3kb *Hin*dIII-*Xho*I *amiE* gene fragment from pJB950 (Cousens *et al*, 1987). This plasmid has *amiE* expressed from its normal promoter with the normal control sequences between the promoter and the gene and so provides a convenient assay for the amount of functional AmiR produced. JA221, pSW35 was made competent and transformed to ampicillin resistance with plasmid pDC5 DNA. The presence of both plasmids in transformants was confirmed by restriction mapping of plasmid DNA isolated from the transformants. Amidase activity was assayed from strain JA221, pSW35, pDC5 under non-inducing, inducing and repressing conditions. The results are shown in Table 3.4. High levels of amidase are produced from this strain under all growth conditions, confirming that AmiR functions independently of amide inducer. This experiment confirms the result with pSW3 and lends further evidence to the

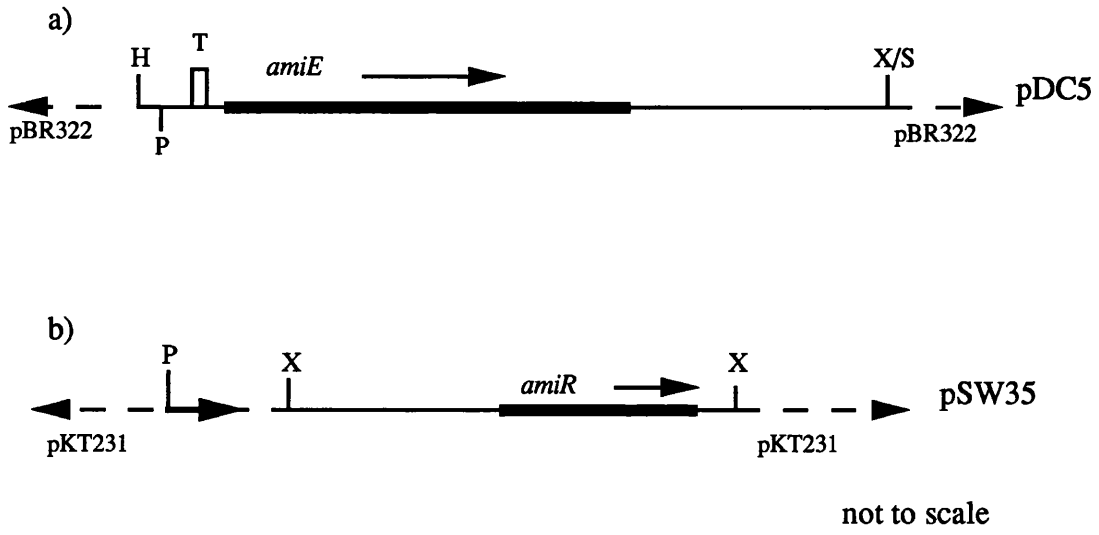


Figure 3.4 a) Restriction map of the 2.3kb *Hin*dIII-*Xho*I PAC433 DNA fragment of plasmid pDC5 (Cousens *et al,* 1987). Abbreviations are as follows: P= *amiE* promoter,T= transcription terminator, H=*Hin*dIII, X/S= the hybrid site generated by ligation of the *Xho*I targets and *Sal* I targets.. The arrow represents the direction of transcription of the *amiE* gene.

b) Restriction map of the 1.5kb *Xho*I *amiR* fragment from pAS20 subcloned into pKT231 to generate plasmid pSW35. Abbreviations are as follows: P= the vector neomycin phosphotransferase II promoter, X= *Xho*I. The arrow represents the direction of transcription of the *amiR* gene.

TABLE 3.3. Amidase activities[a] from pDC5 and pSW35 in *E.coli* strain JA221

	Growth Conditions					
Plasmids	Glucose	Glucose/ Lactamide	Succinate	Succinate/ Lactamide	Succinate/ Butyramide	Mean
pDC5/pSW35	39.7	43.7	39.0	34.1	36.4	38.6

a) Amidase activity was measured as μmoles acetohydroxamate produced per minute per mg bacteria. Values shown are the mean of duplicate assays carried out on at least three separate occasions.

hypothesis that *amiR* expression or activity is regulated by some other factor which is responsive to amide inducer and that this regulation determines the amidase phenotype. The 658bp *Xho*I fragment has been implicated as containing either a *cis* acting element (eg. a promoter/operator or transcription terminator) or/and a gene which could regulate the expression or activity of the *amiR* gene or gene product. To distinguish between these possibilities, two new plasmid constructs were made.

3.4 *In vitro* construction of a deletion and insertion in the DNA sequence upstream of *amiR*

If the region of DNA upstream of *amiR*, including the 655bp *Xho*I fragment, encoded a regulatory gene then deletions and insertions in this region may render the wild type inducible genes constitutive. This hypothesis was tested by (i) making a 250bp deletion immediately upstream of *amiR* in the 1.5kb *Xho*I fragment and (ii) by making an 8bp insertion over 1kb upstream of *amiR* in the 658bp *Xho*I fragment. The latter mutation would be expected to cause a frameshift mutation in a potential regulatory gene.

3.4.1 Construction of plasmids pSW36 and pSW37

Plasmid pSW36 was constructed by digestion of plasmid pAS20 with *Cla*I and then ligating the fragments. The ligation mixture was used to transform *E.coli* JA221 to ampicillin resistance. Plasmid DNA was isolated from transformants and plasmid pSW36 was characterised by restriction mapping. This plasmid has the 250bp *Cla*I fragment deleted, immediately upstream of the *amiR* gene (Figure 3.5). To construct plasmid pSW37, pAS20 was linearised at the unique *Eco*RV target within the 658bp *Xho*I fragment (Figure 3.5). The linearised DNA was ligated with an 8bp *Bam*HI linker with the following sequence - 5'-CGGATCCG-3'. Following ligation, excess linkers were removed by extensive digestion with *Bam*HI to ensure only one linker was inserted into pAS20 in the final construct. Following digestion the DNA was religated and used to transform *E.coli* JA221 to ampicillin resistance. Plasmid DNA was isolated from transformants and recombinant plasmids containing the *Bam*HI linker identified by the presence of a unique *Bam*HI target. A restriction map of pSW37 is shown in Figure 3.5.

3.4.2 Amidase expression from pSW36 and pSW37 in *E.coli*

Amidase activity was measured from pSW36 and pSW37 in *E.coli* JA221 under non-inducing, inducing and repressing conditions. Results are shown in Table 3.5. Both plasmids show low constitutive amidase expression under all growth conditions, which suggests this region of DNA encodes a negative control element, since a frameshift insertion and a 250bp deletion cause constitutive expression. The potential repressor gene was named *amiC*.

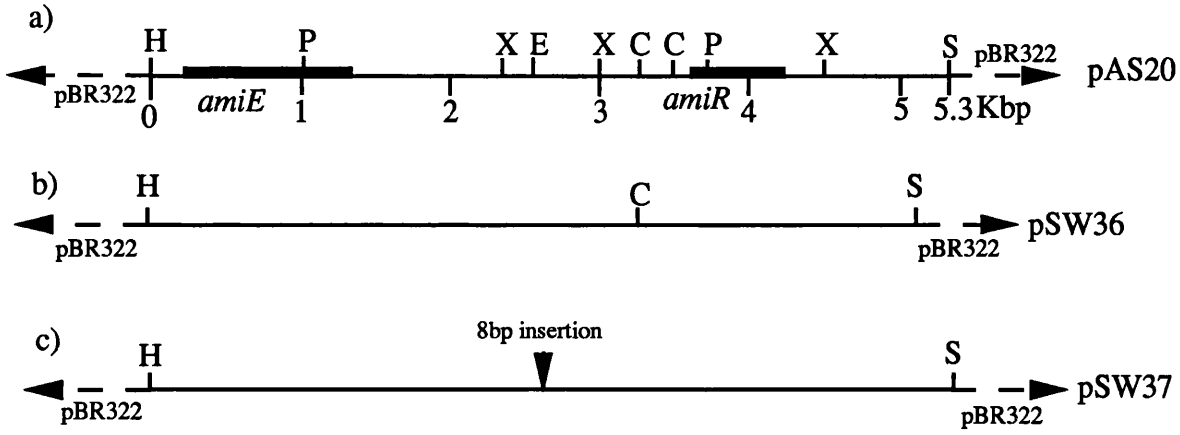


Figure 3.5 a) Restriction map of the *Hin*dIII-*Sal*I fragment of plasmid pAS20. Abreviations for restriction enzyme targets are as follows: H= *Hin*dIII, P= *Pvu*II, X= *Xho*I, E= *Eco*RV, C= *Cla*I and S= *Sal*I.

b) Restriction map of plasmid pSW36, which is identical to plasmid pAS20 except the 250bp *Cla*I fragment is deleted.

c) Restriction map of plasmid pSW37, which is identical to plasmid pAS20 except there is an 8bp *Bam*HI linker subcloned into the unique *Eco*RV target.

TABLE 3.4. Amidase activities[a] from plasmids pAS20, pSW36 and pSW37 in *E.coli* strain JA221.

Plasmid	Growth Conditions					
	Glucose	Glucose/ Lactamide	Succinate	Succinate/ Lactamide	Succinate/ Butyramide	Mean
pAS20	0.0	0.1	0.0	0.0	0.0	0.0
pSW36	2.6	2.8	2.1	2.1	1.9	2.5
pSW37	1.6	1.7	2.5	2.4	1.0	1.8

a) Amidase activity was measured as μmoles acetohydroxamate produced per minute per mg bacteria. Values shown are the mean of duplicate assays carried out on at least three separate occasions.

3.5 DNA sequencing of the region upstream of *amiR*

To confirm the presence of an open reading frame (*amiC*) upstream of *amiR* and to identify the 5' and 3' ends of *amiC*, the DNA sequence from pAS20 between *Xho*I(2.4) and *Pvu*II(3.6) (Figure 3.6) was determined.

3.5.1 Construction of M13 recombinants and sequencing strategy

The sequencing strategy for the region of DNA between *Xho*I and *Pvu*II is shown in Figure 3.6. The arrows represent the direction and extent of DNA sequence obtained from individual clones. Phage SAW1 and SAW4 were constructed by subcloning the 658bp *Xho*I fragment from pAS20 into M13mp18 cut with *Sal*I. Recombinants were identified by restriction mapping of RF DNA isolated from phage infected cells. Phage SAW2 was constructed by isolating a 2.5kb *Xor*II fragment from pAS20, modifying the ends of the

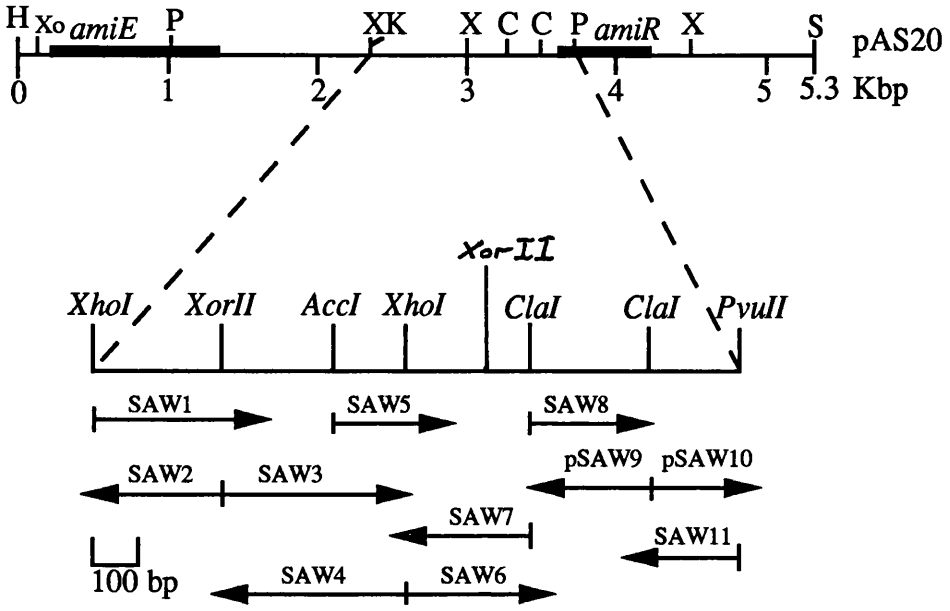


Figure 3.6. The sequencing strategy for the region of DNA between *Xho*I and *Pvu*II. Arrows represent the direction and extent of DNA sequence obtained from each clone. Abbreviations for restriction enzyme targets are as follows: H= *Hin*dIII, P= *Pvu*II, X= *Xho*I, K= *Kpn* I, C= *Cla*I, S= *Sal* , Xo= *Xor* II.

fragment with T4 DNA polymerase to make them blunt ended and then subcloning the fragment into M13mp19 cut with *Sma*I. The orientation of the fragment was determined by restriction mapping of RF DNA isolated from phage infected cells. Phage SAW3 was constructed by isolating the 500bp *Xor*II fragment from pAS20, modifying the ends using T4 DNA polymerase to make them blunt ended and then subcloning the fragment into M13mp18 cut with *Sma*I. The presence of inserts within the recombinant M13 DNA was determined by restriction mapping of the RF DNA. Clones were identified with the insert in both directions by the complementarity test as described by Perbal (1988). Phage SAW5 was constructed by isolating the *Acc*I-*Cla*I fragment from pAS20 and subcloning this fragment into M13mp18 cut with *Acc*I. A clone with the insert in the correct orientation was identified by sequencing several M13 recombinants. Phage SAW6 was constructed by isolating the 1.5kb *Xho*I fragment from pAS20 and subcloning this fragment into M13mp18 cut with *Sal*I. Recombinants containing inserts were identified by restriction mapping of M13 DNA isolated from phage infected cells. Recombinants with inserts in both orientations were identified by the complementarity test.

Phage SAW7 was constructed by isolating the 250bp *Kpn*I-*Cla*I fragment from pAS20 and subcloning this fragment into M13mp19 cut with *Kpn*I and *Acc*I. Recombinant M13 phage containing the insert were identified by linearising RF DNA from potential recombinants with *Hin*dIII and comparing the mobility of this DNA on agarose gels, with M13mp18 RF DNA linearised with *Hin*dIII. Phage SAW8 was constructed by isolating the 250bp *Cla*I fragment from pAS20 and subcloning it into M13mp19 cut with *Acc*I. It was hoped to identify recombinant M13 with the *Cla*I fragment in both orientations but six recombinants were identified containing the *Cla*I fragment and sequenced. In each case the *Cla*I fragment was in the same orientation. To construct SAW9, which would give the DNA sequence reading in the opposite direction to SAW8 it was decided to construct a plasmid containing the *Cla*I fragment which could then be sequenced

in either direction using different primers. The 250bp *Cla*I fragment was isolated from pAS20 and subcloned into pUC18. Recombinant plasmids were identified by restriction mapping. Plasmid pSAW9 was identified and sequenced using universal and reverse sequencing primer to obtain the DNA sequence of both strands of DNA. Plasmid pSAW10 was constructed by digestion of plasmid pSW24 (which contains the 1.5kb *Xho*I fragment subcloned into pUC19 (see chapter four)) with *Hin*dIII and *Cla*I. The cohesive ends produced by these digestions were made blunt-ended using Klenow enzyme. The digested, blunt-ended DNA was subsequently run on an agarose gel and the fragment corresponding to pUC18 + *Cla*I-*Xho*I from pAS20 was isolated and religated. The ligation reaction mixture was used to transform *E.coli* JM101 to ampicillin resistance and plasmid pSAW10 identified by restriction mapping. Phage SAW11 was constructed by isolating the *Hin*dIII-*Pvu*II fragment from pSW24 and subcloning this fragment into M13mp19 cut with *Hin*dIII and *Sma*I. Recombinant phage containing the insert were identified by restriction mapping of RF DNA isolated from phage infected cells.

3.5.2 DNA sequence of the region upstream of *amiR*

The DNA sequence from *Xho*I(2300)-*Pvu*II(3504) was determined using the M13 and plasmid constructs described above. For M13 constructs universal primer was used and for plasmid constructs both universal and reverse sequencing primer were used. The DNA sequence obtained is shown in Figure 3.7

3.5.3 DNA sequence analysis- identification of *amiC* and two *ntrA* dependent promoters

P.aeruginosa generally has a highly biased codon utilisation and this property has previously been used to identify the *amiR* open reading frame (Lowe *et al*, 1989). To identify the *amiC* open reading frame DNA sequence analysis was performed on the sequence between *Xho*I-*Pvu*II using the GCG

XhoI *KpnI*
CTCGAGGTACCG

```
  10          20          30          40          50          60
CTGGCCGAGCATCTGCTCGATCACCACCAGCCGGGCGACGGGAACTGCACGATCTACCTGG

          80          90          100         110         120         130
CGAGCCTGGAGCACGAGCGGGTTCGCTTCGTACGGCGCTGAGCGACAGTCACAGGAGAGGA

          140         150         160         170         180
AACGG ATG GGA TCG CAC CAG GAG CGG CCG CTG ATC GGC CTG CTG TTC TCC GAA
      Met Gly Ser His Gln Glu Arg Pro Leu Ile Gly Leu Leu Phe Ser Glu

      190         200         210         220         230
ACC GGC GTC ACC GCC GAT ATC GAG CGC TCG CAC GCG TAT GGC GCA TTG CTC GCG
Thr Gly Val Thr Ala Asp Ile Glu Arg Ser His Ala Tyr Gly Ala Leu Leu Ala

    240         250         260         270         280         290
GTC GAG CAA CTG AAC CGC GAG GGC GGC GTC GGC GGT CGC CCG ATC GAA ACG CTG
Val Glu Gln Leu Asn Arg Glu Gly Gly Val Gly Gly Arg Pro Ile Glu Thr Leu

          300         310         320         330         340
TCC CAG GAC CCC GGC GGC GAC CCG GAC CGC TAT CGG CTG TGC GCC GAG GAC TTC
Ser Gln Asp Pro Gly Gly Asp Pro Asp Arg Tyr Arg Leu Cys Ala Glu Asp Phe

    350         360         370         380         390
ATT CGC AAC CGG GGG GTA CGG TTC CTC GTG GGC TGC TAC ATG TCG CAC ACG CGC
Ile Arg Asn Arg Gly Val Arg Phe Leu Val Gly Cys Tyr Met Ser His Thr Arg

          410         420         430         440         450
AAG GCG GTG ATG CCG GTG GTC GAG CGC GCC GAC GCG CTG CTC TGC TAC CCG ACC
Lys Ala Val Met Pro Val Val Glu Arg Ala Asp Ala Leu Leu Cys Tyr Pro Thr

      460         470         480         490         500
CCC TAC GAG GGC TTC GAG TAT TCG CCG AAC ATC GTC TAC GGC GGT CCG GCG CCG
Pro Tyr Glu Gly Phe Glu Tyr Ser Pro Asn Ile Val Tyr Gly Gly Pro Ala Pro

  510         520         530         540         550         560
AAC CAG AAC AGT GCG CCG CTG GCG GCG TAC CTG ATT CGC CAC TAC GGC GAG CGG
Asn Gln Asn Ser Ala Pro Leu Ala Ala Tyr Leu Ile Arg His Tyr Gly Glu Arg

          570         580         590         600         610
GTG GTG TTC ATC GGC TCG GAC TAC ATC TAT CCG CGG GAA AGC AAC CAT GTG ATG
Val Val Phe Ile Gly Ser Asp Tyr Ile Tyr Pro Arg Glu Ser Asn His Val Met

    620         630         640         650         660
CGC CAC CTG TAT CGC CAG CAC GGC GGC ACG GTG CTC GAG GAA ATC TAC ATT CCG
Arg His Leu Tyr Arg Gln His Gly Gly Thr Val Leu Glu Glu Ile Tyr Ile Pro

          680         690         700         710         720
CTG TAT CCC TCC GAC GAC GAC TTG CAG CGC GCC GTC GAG CGC ATC TAC CAG GCG
Leu Tyr Pro Ser Asp Asp Asp Leu Gln Arg Ala Val Glu Arg Ile Tyr Gln Ala

      730         740         750         760         770
CGC GCC GAC GTG GTC TTC TCC ACC GTG GTG GGC ACC GGC ACC GCC GAG CTG TAT
Arg Ala Asp Val Val Phe Ser Thr Val Val Gly Thr Gly Thr Ala Glu Leu Tyr

  780         790         800         810         820         830
CGC GCC ATC GCC CGT CGC TAC GGC GAC GGC AGG CGG CCG CCG ATC GCC AGC CTG
Arg Ala Ile Ala Arg Arg Tyr Gly Asp Gly Arg Arg Pro Pro Ile Ala Ser Leu

          840         850         860         870         880
ACC ACC AGC GAG GCG GAG GTG GCG AAG ATG GAG AGT GAC GTG GCA GAG GGG CAG
Thr Thr Ser Glu Ala Glu Val Ala Lys Met Glu Ser Asp Val Ala Glu Gly Gln

    890         900         910         920         930
GTG GTG GTC GCG CCT TAC TTC TCC AGC ATC GAT ACG CCC GCC AGC CGG GCC TTC
Val Val Val Ala Pro Tyr Phe Ser Ser Ile Asp Thr Pro Ala Ser Arg Ala Phe

          950         960         970         980         990
GTC CAG GCC TGC CAT GGT TTC TTC CCG GAG AAC GCG ACC ATC ACC GCC TGG GCC
Val Gln Ala Cys His Gly Phe Phe Pro Glu Asn Ala Thr Ile Thr Ala Trp Ala

     1000        1010        1020        1030        1040
GAG GCG GCC TAC TGG CAG ACC TTG TTG CTC GGC CGC GCC GCG CAG GCC GCA GGC
Glu Ala Ala Tyr Trp Gln Thr Leu Leu Leu Gly Arg Ala Ala Gln Ala Ala Gly

 1050        1060        1070        1080        1090        1100
AAC TGG CGG GTG GAA GAC GTG CAG CGG CAC CTG TAC GAC ATC GAC ATC GAC GCG
Asn Trp Arg Val Glu Asp Val Gln Arg His Leu Tyr Asp Ile Asp Ile Asp Ala
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        1110          1120          1130          1140          1150
CCA CAG GGG CCG GTC CGG GTG GAG CGC CAG AAC AAC CAC AGC CGC CTG TCT TCG
Pro Gln Gly Pro Val Arg Val Glu Arg Gln Asn Asn His Ser Arg Leu Ser Ser

   1160          1170          1180          1190          1200
CGC ATC GCG GAA ATC GAT GCG CGC GGC GTG TTC CAG GTC CGC TGG CAG TCG CCC
Arg Ile Ala Glu Ile Asp Ala Arg Gly Val Phe Gln Val Arg Trp Gln Ser Pro

           1220          1230          1240          1250          1260
GAA CCG ATT CGC CCC GAC CCT TAT GTC GTC GTG CAT AAC CTC GAC GAC TGG TCC
Glu Pro Ile Arg Pro Asp Pro Tyr Val Val Val His Asn Leu Asp Asp Trp Ser

      1270          1280          1290          1300          1310
GCC AGC ATG GGC GGG GGA GCG CTC CCA TG AGC GCC ACC TCG CTG CTC GGC AGC
Ala Ser Met Gly Gly Gly Ala Leu Pro -- -
                                  Met Ser Ala Asn Ser Leu Leu Gly Ser

  1320          1330          1340          1350          1360
CTG CGC GAG TTG CAG GTG CTG GTC CTC AAC CCG CCG GGG GAG GTC AGC GAC GCC
Leu Arg Glu Leu Gln Val Leu Val Leu Asn Pro Pro Gly Glu Val Ser Asp Ala

              PvuII
CTG GTC TTG CAG CTG
Leu Val Leu Gln Leu
```

Figure 3.7. DNA sequence from *Xho*I(2400)-*Pvu*II(3700). The *Xho*I, *Kpn*I and *Pvu*II targets are underlined and labelled. A translation of the *amiC* open reading frame is shown under the DNA sequence. The two potential *ntrA* dependent promoters are underlined and the conserved nucleotides at -12 and -24 are shown in bold. The *amiC* ribosome binding site is underlined between positions 121 and 126. The unique *EcoRV* target, used to make the frameshift mutation in *amiC*, is underlined between positions 198 and 203.

software package (version 6.1) from the University of Wisconsin (Devereux *et al*, 1984). The results of this analysis are shown in Figure 3.8. Each panel represents one of the three reading frames. The codon preference statistic (Gribskov *et al*, 1984) and third position GC bias (Bibb *et al*, 1984) were calculated over a window of 25 codons with a codon usage table obtained using published *P.aeruginosa* sequences from the EMBL library, release 14.0, plus the *amiE* sequence. The reference lines at codon preference 0.84 and GC bias of 0.65 show values for random sequence with the same base composition. Rare codons, with a preference below 0.1 are indicated by vertical bars underneath each panel. Open reading frames are identified underneath each panel by horizontal boxes (|———|).

From the increase in codon preference statistic and third position GC bias and infrequent use of rare codons an open reading frame corresponding to *amiC* was identified from bases 136-1295 in reading frame 2. The position of the *Cla*I deletion in pSW36 is between residues 911-1186 and leads to an in frame deletion of part of the C-terminal region of AmiC. The 8bp *Bam*HI linker in pSW37 causes a frameshift mutation at the 5' end of the *amiC* open reading frame. The *amiC* open reading frame codes for a 385 amino acid protein with a predicted molecular weight of 42,834 Da. A translation of *amiC* is shown under the corresponding DNA sequence in Figure 3.7.

Interestingly the stop codon for *amiC* and the start codon for *amiR* overlap by two base pairs to give the sequence ATGA. The absence of an intergenic region between *amiC* and *amiR* suggests that the two genes may form part of an operon and their expression may be co-ordinately regulated.

Part of the sequence (*Xho*I(3000)-*Pvu*II(3700)) for *amiC* from the constitutive mutant PAC433 has previously been determined by Nick Lowe (personal communication) and a comparison with the wild type sequence for this region has

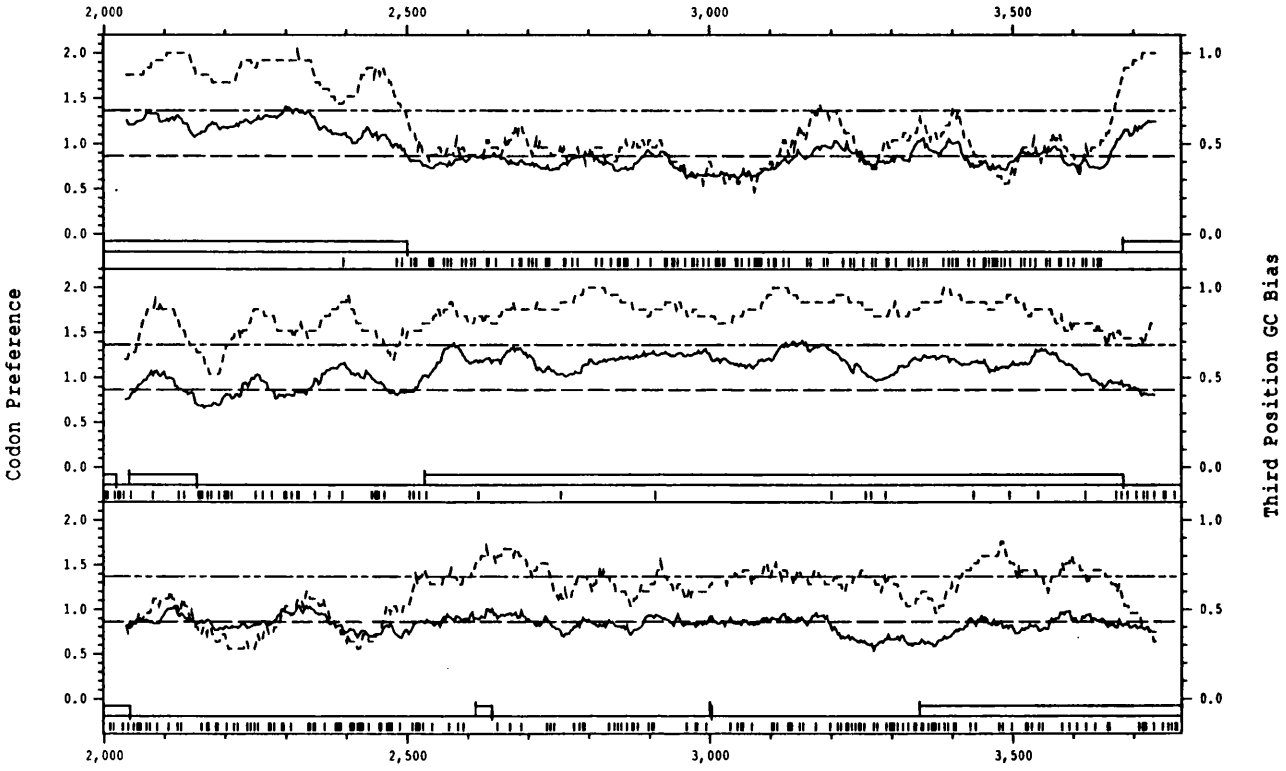

Figure 3.8. Codon preference analysis for the *amiC* gene. The sequence is numbered from the unique *Hin*dIII target upstream of *amiE*. Each panel represents one reading frame, RF(1) top, RF(2) middle and RF(3) bottom. The continuous line (———) shows the codon preference statistic, the dashed line (-----) shows the third position GC bias and vertical bars below each panel show rare codons. The reference lines at codon preference of 0.84 and GC bias of 0.65 show the values for random sequence with the same base composition. Open reading frames are indicated by boxes at the bottom of each panel. The *amiC* open reading frame is seen in panel two between positions 2528 and 3687. The 3' end of the open reading frame in panel one corresponds to the *amiY* gene (see Chapter six).

identified three point mutations in *amiC* from PAC433. The mutations from pAS20 to pJB950 are as follows:- 1. C > A at 689 giving an Asp > Glu mutation; 2. C > T at 898 giving a Pro > Leu mutation; 3. G > C at 1281 giving an Ala > Pro mutation. Whether these mutations are the reason for this strain being constitutive is impossible to tell at this stage, primarily because the sequence of the 658bp *Xho*I fragment from PAC433 has not been completely determined therefore we do not have the full *amiC* sequence from PAC433.

In the *amiC* leader region (121-126) is a DNA sequence complementary to the 3' end of the *P.aeruginosa* 16s rRNA (Toschka *et al*, 1988) which presumably acts as the ribosome binding site (RBS). Upstream of the RBS are two blocks of sequence 8-24 and 65-82 which show homology to the consensus *ntr*A dependent promoter. A comparison of these potential promoters with known *ntr*A dependent promoters is shown in Figure 3.9. The critical features of this type of promoter are two blocks of sequence (GG and GC) spaced exactly 10bp apart at positions -24 and -12 upstream from the transcription start point. Whether one or both of these promoter elements represents the *amiC/amiR* promoter is impossible to tell at this stage and a more thorough investigation and discussion of these promoters will be left until Chapter 6.

3.5.4 Codon utilisation for *amiC*

A codon utilisation table for *amiC* calculated from the derived amino acid sequence is shown in Table 3.6. Codon usage for *amiC* is similar to, but less extreme than that for *amiE* (Brammar *et al*, (1987) and *amiR* (Lowe *et al*, 1989). 46 of the 61 possible sense codons are used and only 11% show A or T in the third position. For some amino acids a single codon is used exclusively, UUC for Phe(11), AAC for Asn(11) and AAG for Lys(2).

nifF	GTGCAAAGCAACCTGGCACAGCCTTCGCTATACCCCTGCGA
nifL	ATCACGCCGATAAGGGCGCACGGTTTGCATGGTTATCACCG
nifE	AAGGCTCCGCTTCTGGAGCGCGAATTGCATCTTCCCCCTCA
nifM	AGCCAGCCGTGGCTGGCCGGAAATTTGCAATACAGGGATAG
nifB	CGAAATTAACCTCTGGTACAGCATTTGCAGCAGGAAGGTAT
nifH	AAACAGGCACGGCTGGTATGTTCCCTGCACTTCTCTGCTGG
nifU	TAATTTTATTCTCTGGTATCGCAATTGCTAGTTCGTTATCG
xylCAB	TCGGTATAAGCAATGGCATGGCGGTTGCTAGCTATACGAGA
xylS	TCTTCGTTCTGCTTGGCGTTATTTTTGCTTGGAAAAGTGG
PAK	TGTGAACGGTATTTGGCACTCGAATTGCTTGGTAGGGTTA
CPG2	AAGGCACCGCAGTGGCACTCGAATTGCTATAAGAACCATG
glnAp2	GCAATTTAAAAGCTGGCACAGATTTCGCTTTATCTTTTTTA

nif consensus		TGGYAYRNNNNTTGCA
Pseudomonas consensus	T/G	TGGCAT(T/G)NNNNTTGCT(A/T)GNNANA

amiCR1	CTCGAGGTACCGCTGGCCGAGCATCTGCTCGATCACCACCA
amiCR2	TGCACGATCTACCTGGCGAGCCTGGAGCACGAGCGGGTTCG

Figure 3.9. A comparison of the two potential promoters upstream of *amiC* with known *ntrA* dependent promoters. Sequences shown are from -40 to +1. The top block of sequences are the *nif* promoter sequences from *Klebsiella pneumoniae*. The central block are *Pseudomonas* promoters recognised by this sigma factor. The *glnAp2* promoter is the σ^{54}-dependent promoter for glutamine synthetase. The *Pseudomonas* consensus sequence is taken from Deretic *et al*, 1989. *amiCR1* is the 5' promoter sequence and *amiCR2* the 3' sequence. The conserved nucleotides at -12 and -24 are shown in bold. The sequences in *amiCR1* and *amiCR2* which are homologous with the *Pseudomonas* consensus sequence, are shown in bold. Sequences in the *nif* and *Pseudomonas* promoter block, which have homology with *amiC*R1 are underlined.

Table 3.5 - Codon utilisation for the *amiC* gene.

Codon	Amino acid	Number	%	Codon	Amino acid	Number	%
TTT	Phe	0	0.0	ATT	Ile	4	1.0
TTC	Phe	11	2.9	ATC	Ile	16	4.2
TTA	Leu	0	0.0	ATA	Ile	0	0.0
TTG	Leu	4	1.0	ATG	Met	6	1.6
TCT	Ser	1	0.3	ACT	Thr	0	0.0
TCC	Ser	6	1.6	ACC	Thr	11	2.9
TCA	Ser	0	0.0	ACA	Thr	0	0.0
TCG	Ser	7	1.8	ACG	Thr	4	1.0
TAT	Tyr	8	2.1	AAT	Asn	0	0.0
TAC	Tyr	13	3.4	AAC	Asn	11	2.9
TAA	Stop	0	0	AAA	Lys	0	0.0
TAG	Stop	0	0	AAG	Lys	2	0.5
TGT	Cys	0	0	AGT	Ser	2	0.5
TGC	Cys	4	1.0	AGC	Ser	7	1.8
TGA	Stop	1	--	AGA	Arg	0	0.0
TGG	Trp	5	1.3	AGG	Arg	1	0.3
CTT	Leu	0	0.0	GTT	Val	0	0.0
CTC	Leu	7	1.8	GTC	Val	13	3.4
CTA	Leu	0	0.0	GTA	Val	1	0.3
CTG	Leu	15	3.9	GTG	Val	19	4.9
CCT	Pro	2	0.5	GCT	Ala	0	0.0
CCC	Pro	6	1.6	GCC	Ala	18	4.7
CCA	Pro	2	0.5	GCA	Ala	3	0.8
CCG	Pro	17	4.4	GCG	Ala	18	4.7
CAT	His	3	0.8	GAT	Asp	3	0.8
CAC	His	8	2.1	GAC	Asp	19	4.9
CAA	Gln	1	0.3	GAA	Glu	7	1.8
CAG	Gln	15	3.9	GAG	Glu	19	4.9
CGT	Arg	1	0.3	GGT	Gly	3	0.8
CGC	Arg	22	5.7	GGC	Gly	23	6.0
CGA	Arg	0	0.0	GGA	Gly	2	0.5
CGG	Arg	11	2.9	GGG	Gly	4	1.0

The percentage values refer to the percentage of the total 385 amino acids used.

3.6 Construction and use of a promoter probe vector

The experiments described above have identified a negative control element *amiC* . It has also been shown by sequence homology that the 658bp *Xho*I fragment contains two potential *ntrA* dependent promoters upstream of *amiC*. To investigate the promoter activity in the 658bp *Xho*I fragment more thoroughly and to test whether *amiC* might affect expression from this/these promoters it was decided to construct a promoter probe vector whereby a promoterless gene whose expression could be easily assayed was placed downstream of i) the 658bp *Xho*I fragment ii) a 2kb fragment from *Sma*I(1900) to *Pvu*II (3700) which encompassed 500bp of DNA upstream of the 658bp *Xho*I fragment, the whole of the 658bp *Xho*I fragment and the whole of the *amiC* coding sequence. This latter construct would in effect be an operon fusion between the *amiC* gene plus its promoter/control elements with an assayable gene downstream and it was hoped this construct would test whether *amiC* formed part of an autogeneous control circuit regulating expression of itself and *amiR* from the promoters upstream of *amiC*. It was decided to construct a promoter probe vector for these experiments based on a promoterless *amiE* gene as it was easy to isolate a fragment containing the whole of the *amiE* gene without its promoter and amidase is a simple enzyme to assay.

3.6.1 Construction of plasmid pSW7

Plasmid pSW7 was initially constructed for use as a promoter probe vector as follows: A promoterless *amiE* gene fragment was isolated from pAS20 on a 1.7kb *Sma*I fragment. *Hin*dIII linkers were ligated to this fragment, and excess linkers subsequently removed by extensive digestion with *Hin*dIII. The fragment having *Hin*dIII cohesive ends was ligated into pBR322 cut with *Hin*dIII. The ligation mixture was used to transform *E.coli* JA221 to ampicillin resistance and pSW7 was identified by restriction mapping of plasmid DNA isolated from transformants. A restriction map of pSW7 and the direction of transcription of the *amiE* gene are shown in Figure 3.10. This vector was not subsequently used for experiments because there were few cloning sites available for insertion of test fragments. An improved promoter probe vector was constructed which would have wider use and construction of this vector is described below.

3.6.2 Construction of plasmids pSW9 and pSW10

To construct a promoter probe vector with several unique cloning sites for test fragments a derivative of pBR322 (pSW9) was first constructed before insertion of the *amiE* gene fragment. To construct pSW9, M13mp18 and pBR322 were digested with *Hin*dIII and *Eco*RI and the mixture of fragments ligated. The ligation mixture was used to transform *E.coli* JA221 to ampicillin resistance and plasmid pSW9 was identified by restriction mapping of plasmid DNA isolated from transformants. This plasmid has the multiple cloning site from M13mp18 subcloned between *Eco* RI and *Hin*dIII in pBR322. Subsequently plasmid pSW9 was linearised with *Hin*dIII and the promoterless *amiE* *Hin*dIII fragment from pSW7 was subcloned into pSW9. This plasmid was called pSW10 and the construction of this plasmid and a restriction map are shown in Figure 3.10. pSW10 has the following unique restriction targets upstream of the *amiE* gene available for cloning test fragments:- *Eco*RI, *Sst*I, *Kpn*I, *Sma*I, *Xma*I, *Xba*I. The *Sma*I target is particularly useful because any blunt ended fragment may be cloned into this restriction site. Plasmid pSW10 was used for subsequent experiments with test fragments of DNA.

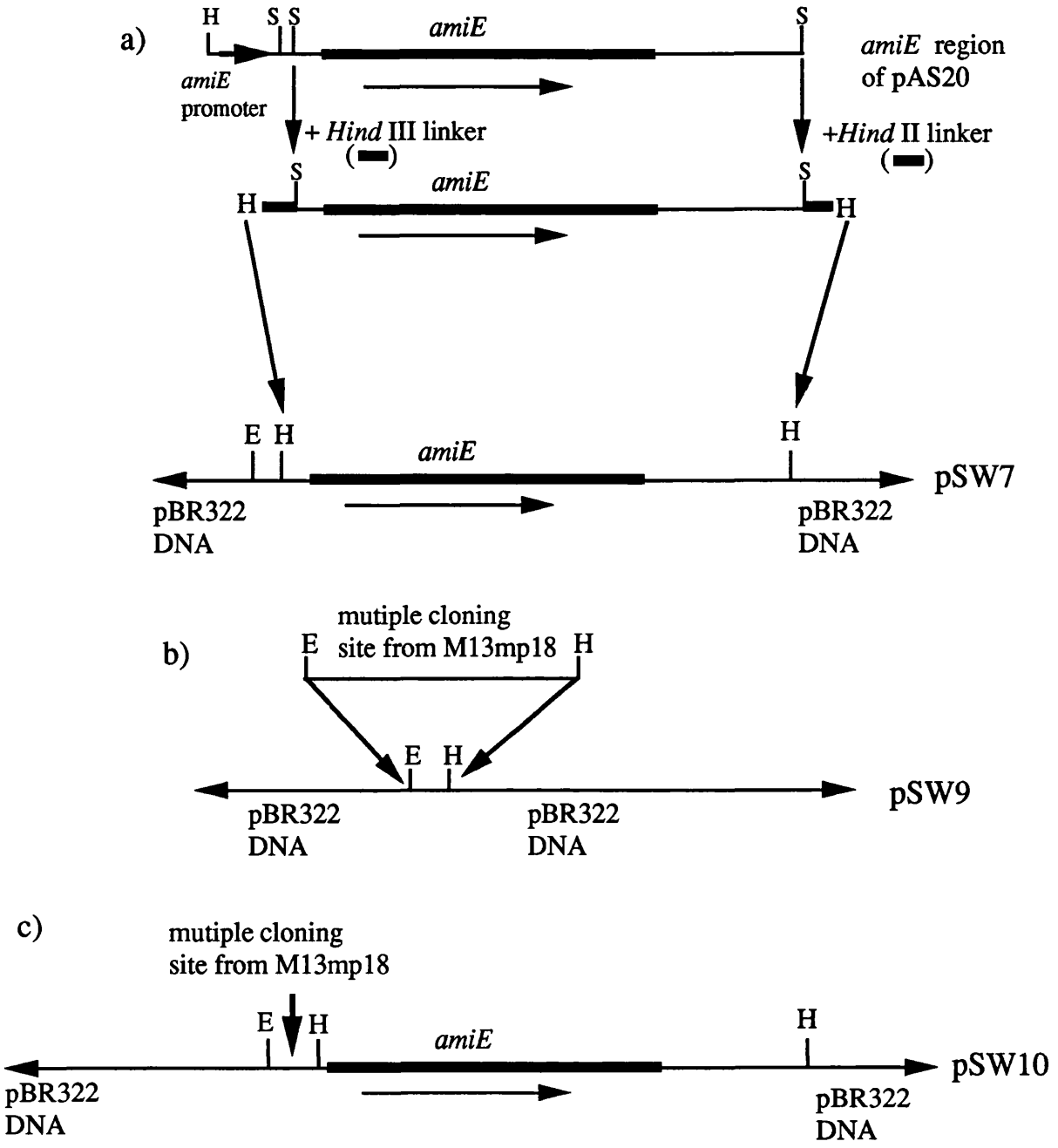

Figure 3.10. a) Construction of plasmid pSW7. The 1.7kb *Sma* I fragment from pAS20 containing a promoterless *amiE* gene was modified by the addition of synthetic *Hind* III linkers and then subcloned into the unique *Hind* III target of pBR322. The arrow indicates the direction of transcription of the *amiE* gene.

b) Construction of plasmid pSW9. The multiple cloning site from M13mp18 was subcloned into the unique *Eco* RI and *Hind*III targets of pBR322 to generate plasmid pSW9.

c) Restriction map of pSW10. The promoterless *Hind* III *amiE* fragment from pSW7 was subcloned into the unique *Hind* III target of pSW9 to generate plasmid pSW10 which now contains a multiple cloning site upstream of a promoterless *amiE* gene.

Abbreviations for restriction enzyme targets are as follows:- H= *Hind* III, E= *Eco* RI, S= *Sma* I. The diagrams are not drawn to scale.

3.6.3 Construction of plasmids pSW11-pSW16

To confirm the 658bp *Xho*I fragment had promoter activity this fragment was cloned into the promoter probe vector pSW10 as follows. The 658bp *Xho*I fragment from pAS20 was subcloned into the *Sal*I target of pUC18. Two plasmids were identified with the 658bp *Xho*I fragment in both orientations. These plasmids were called pSW20 and pSW21 and restriction maps are shown in Figure 3.11. Subsequently the 658bp *Xho*I fragment was isolated from pSW20 by digestion with *Kpn*I. This *Kpn*I fragment was then subcloned into pSW10 and two plasmids were identified with the *Kpn*I fragment in both orientations. Plasmid pSW12 has the 680bp *Kpn*I fragment inserted upstream of *amiE* in the normal orientation and pSW11 has this fragment in the opposite orientation.

To test whether *amiC* was involved in an autogeneous control circuit as described in section 3.6, derivatives of pSW10 were made carrying either the PAC1 or PAC433 *Sma*I-*Pvu*II fragment encoding *amiC* and upstream control sequences. Plasmids pSW13 and pSW14 were constructed by isolating the 2.0kb *Sma*I-*Pvu*II fragment from pAS20 and subcloning it into pSW10 cut with *Sma*I. Plasmid pSW13 has this fragment inserted in the normal orientation for expression and pSW14 has the fragment in the opposite orientation. Plasmids pSW15 and pSW16 were constructed by isolating the 2.0kb *Sma*I-*Pvu*II fragment from pJB950, which carries PAC433 DNA. Plasmid pSW15 has the test fragment inserted in the normal orientation for expression and pSW16 has the fragment inserted in the opposite orientation. The derivation of all the plasmids described in this section and their restriction maps are shown in Figures 3.11 and 3.12.

3.6.4 Amidase expression from plasmids pSW11-16 in *E.coli*

Amidase activity was measured from plasmids pSW11-pSW16 in *E.coli* JA221 under non-inducing, inducing and repressing growth conditions. Results of assays with plasmids pSW10,pSW11 and pSW12 are shown in Table 3.6. Plasmid pSW10 shows a basal level of amidase expression of approximately 0.2 units. This low level of activity could represent residual transcription from the tetracycline resistance promoter of pBR322.

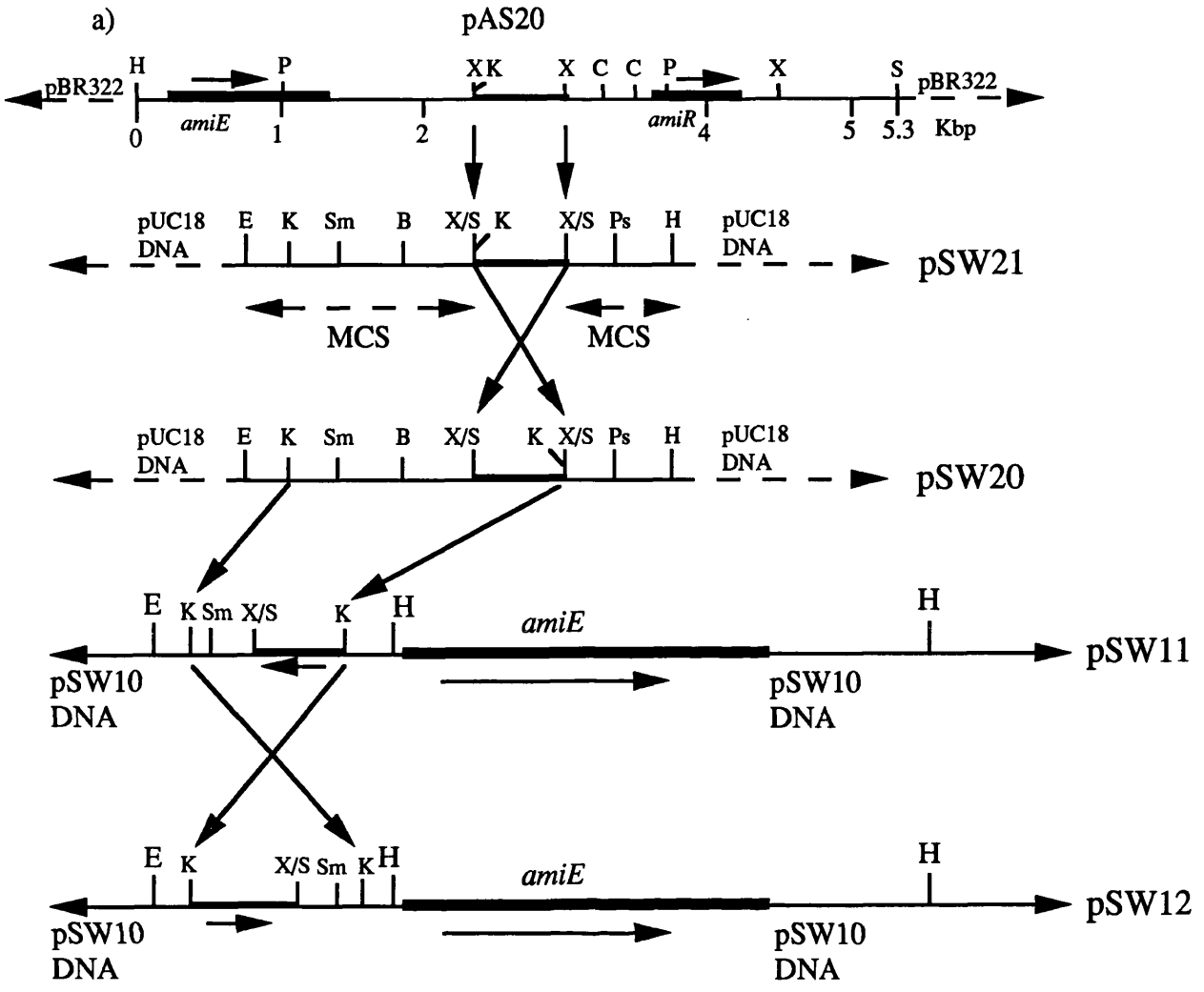

Figure 3.11. Construction of plasmids pSW20, pSW21, pSW11 and pSW12 and their restriction maps. Plasmids pSW20 and pSW21 were constructed by subcloning the 658bp *Xho* I fragment from pAS20 into the multiple cloning site of pUC18. The arrows underneath the inserted *Xho* I fragment indicate whether the fragment is in the normal or reverse orientation. To construct plasmids pSW11 and pSW12, the *Kpn* I fragment from pSW20, containing the 658bp *Xho* I fragment was subcloned into the promoter probe vector pSW10, to generate plasmids pSW11 and pSW12. pSW11 has the *Kpn* I fragment in the reverse orientation and pSW12 has the *Kpn* I fragment in the normal oreintation for expression. Abbreviations are as follows:- H= *Hind* III, P= *Pvu* II, E= *Eco* RI, K= *Kpn* I Sm= *Sma* I, Ps= *Pst* I, B= *Bam* HI, X/S= the hybrid site generated by ligation of *Xho* I and *Sal* I targets.

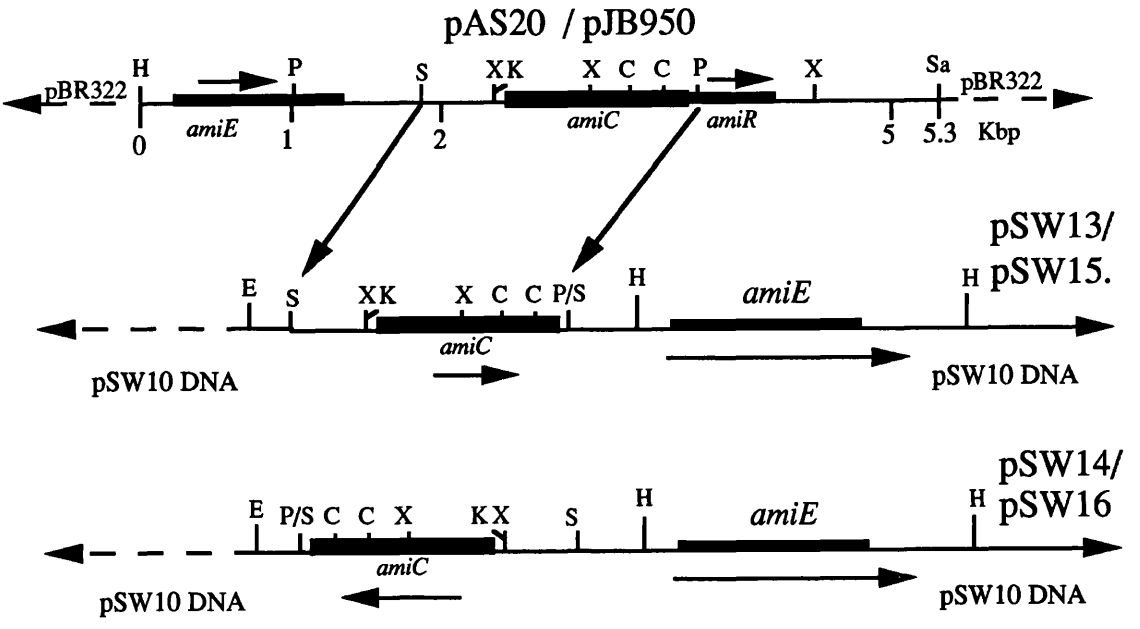

Figure 3.12. Construction of plasmids pSW13, pSW14, pSW15 and pSW16. Plasmids pSW13 and pSW14 contain the 2.0kb *Sma* I- *Pvu* II fragment from pAS20 and plasmids pSW15 and pSW16 contain the same fragment from pJB950. With all four plasmids the test fragment has been inserted into the *Sma* I target of pSW10. In pSW13 and pSW15 the fragment is in the normal orientation for *amiC* expression and in plasmids pSW14 and pSW16 it is in the reverse orientation. Abbreviations are as follows:- H= *Hind* III, P= *Pvu* II, E= *Eco* RI, K= *Kpn* I S= *Sma* I, C= *Cla* I, X= *Xho* I, P/S= the hybrid site generated by ligation of *Pvu* II and *Sma* I restriction targets, Sa = *Sal* I.

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TABLE 3.6. Amidase activities[a] from plasmids pSW10, pSW11 and pSW12 in *E.coli* strain JA221.

	Growth Conditions					
Plasmid	Glucose	Glucose/ Lactamide	Succinate	Succinate/ Lactamide	Succinate/ Butyramide	Mean
pSW10	0.1	0.2	0.3	0.1	0.2	0.2
pSW11	0.0	0.0	0.0	0.1	0.0	0.0
pSW12	0.5	0.5	0.65	0.48	0.26	0.5

a) Amidase activity was measured as μmoles acetohydroxamate produced per minute per mg bacteria. Values shown represent the mean of assays carried out in duplicate on at least three separate occasions.

****

Plasmid pSW11 which has the 658bp *Xho*I fragment in the reverse orientation, shows no detectable amidase activity. Plasmid pSW12 with the 658bp *Xho*I fragment in the correct orientation, shows constitutive amidase expression with an average activity of 0.5 units This level of expression was disappointingly low and suggested that the promoter(s) within the 658bp *Xho*I fragment were extremely weak, or were not efficiently recognised in *E.coli*. Plasmids pSW13-pSW16 showed no detectable amidase expression in *E.coli*, under any growth conditions, presumably because the promoter element within the 658bp *Xho*I fragment was functioning very inefficiently. It was hoped that if *amiC* formed part of an autogeneous control circuit regulating it's own expression and that of *amiR*, then with plasmid pSW13 we would see inducible amidase expression and with plasmid pSW15 containing DNA from a constitutive mutant we might see constitutive amidase expression.

As the promoter element(s) appears to function inefficiently in *E.coli* these hypotheses were not tested by this experiment. Poor expression of *P.aeruginosa* genes in *E.coli* is not an uncommon phenomenon and has been attributed in part to the poor recognition of *Pseudomonas* promoters in *E.coli*. To try and get round this problem it was decided to construct broad host range homologues of plasmids pSW11-16 which could then be used in *P.aeruginosa*.

3.6.5 Construction of plasmids pSW11B-16B

To construct broad host range plasmids analogous to plasmids pSW11-16, plasmid pKT240 was used. Firstly the test fragments were cloned into this plasmid and subsequently the *amiE* gene was placed downstream of these fragments. To do this the *Hin*dIII-*Eco*R1 fragments, containing the test fragments, were isolated from plasmids pSW11-16 and subcloned into the *Hin*dIII and *Eco*-RI sites of plasmid pKT240. Recombinant plasmids were identified by restriction mapping with *Pst*I. The newly constructed plasmids were named after the parent plasmids with the addition of the letter B eg. pSW11B, pSW12B etc. Restriction maps of these plasmids are shown in Figure 3.13

3.6.6 Construction of plasmids pSW113, pSW115 and pSW116

To subclone the *amiE* gene downstream of the test fragments, plasmids pSW11B-16B were linearised with *Hin*dIII and the 1.7kb *Hin*dIII *amiE* fragment from pSW7 was subcloned into this site. The orientation of the *amiE* gene was determined by restriction mapping with *Pst*I. Unfortunately only three new recombinant plasmids containing the *amiE* gene in the normal orientation were identified, plasmids pSW113, pSW115 and pSW116. Restriction maps of these plasmids are shown in Figure 3.13

3.6.7 Amidase expression from plasmids pSW113, 115 and pSW116 in *P.aeruginosa*

Plasmids pSW113, pSW115 and pSW116 were mobilised into the Ami deletion strain PAC452, by triparental matings with the helper plasmid pRK2013. Carbenicillin resistant transconjugants were isolated and assayed for amidase

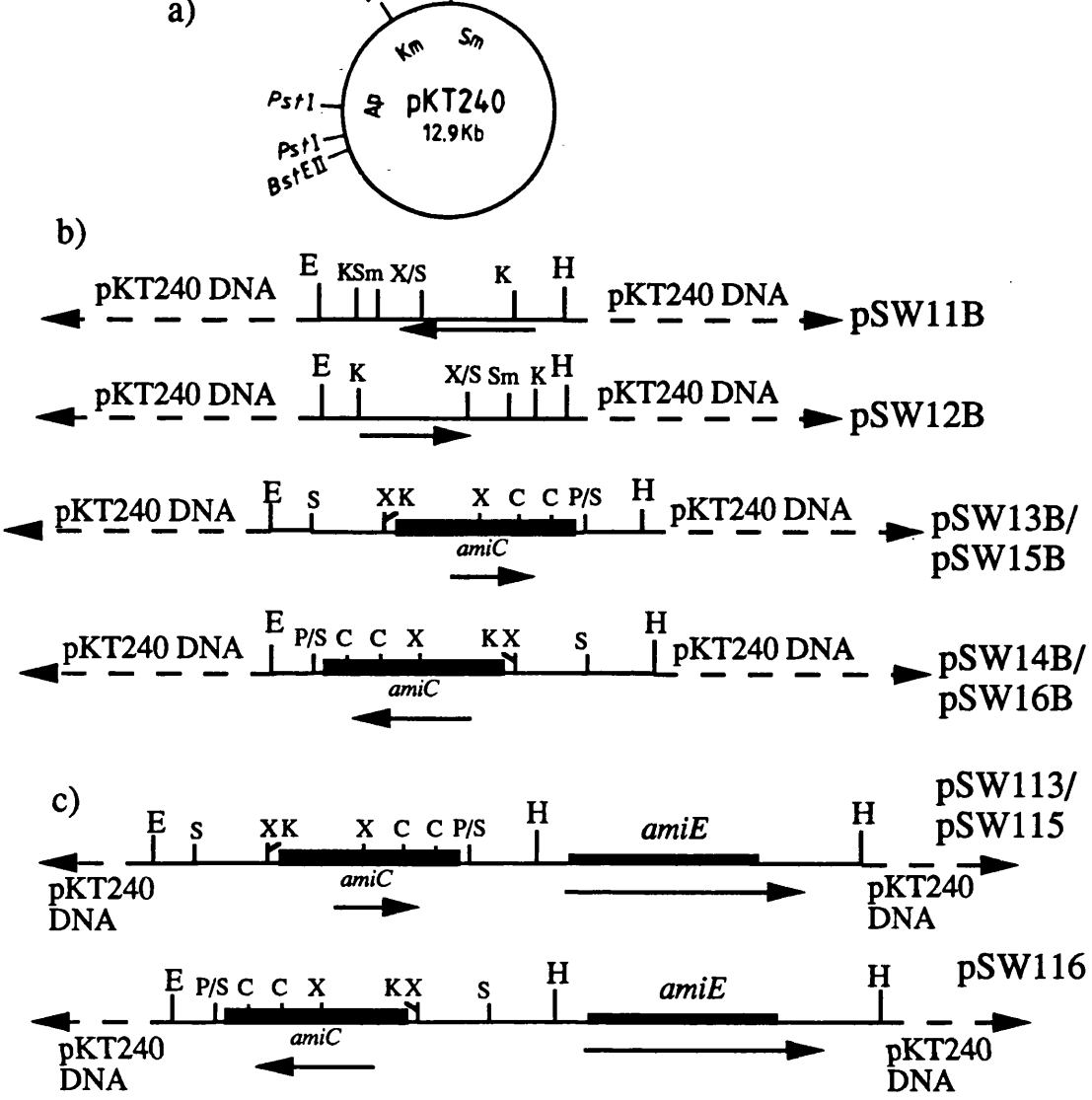

Figure 3.13. Construction of plasmids pSW11B-16B, pSW113, pSW115 and pSW116 and their restriction maps. a) Restriction map of the broad host range cloning vector pKT240. b) Plasmids pSW11B-16B were constructed by isolating the *Hind* III-*Eco*RI fragments from pSW11-16 (see Fig. 3.11 & 3.12) and subcloning the fragments into the *Hind* III and *Eco* RI sites of pKT240. c) Plasmids pSW113 and pSW115 were constructed by isolating the promoterless *amiE* *Hind* III fragment from pSW10 and subcloning this fragment into the unique *Hind* III targets of pSW13B and pSW15B respectively. Thus pSW113 contains a wild type *amiC* gene test fragment and pSW115 contains a constitutive mutant test fragment. pSW116 was constructed in a manner similar to pSW113 and pSW115 and consists of pSW16B with the promoterless *amiE* gene cloned downstream. Abbreviations are as follows:- H= *Hind* III, P= *Pvu* II, E= *Eco* RI, K= *Kpn* I S= *Sma* I, C= *Cla* I, X= *Xho* I, P/S= the hybrid site generated by ligation of *Pvu* II and *Sma* I restriction targets.

activity. None of these plasmid constructs gave detectable amidase activity, under any growth conditions. This would suggest that even in the normal host the potential promoter elements in the 658bp *Xho*I fragment are very weak and do not direct detectable transcription of *amiE* in these constructs. Despite the failure to show amidase expression with any of these promoter probe vector constructs there is no doubt that pSW10 is a useful vector, with a reporter gene that is even easier to assay than the commonly used *lacZ* gene.

3.7 Summary

The promoter for *amiR* had previously been identified within the 950bp *Kpn*I-*Cla*I fragment upstream of *amiR*, by Cousens *et al* (1987). With the construction of plasmids pSW1-5 this promoter element was further localised to the 658bp *Xho*I fragment. With plasmids pSW3 and pSW5, missing the 658bp *Xho*I fragment, it was found that there was increased amidase expression. Cousens *et al* (1987) had similarly shown that *Bal*31 generated deletions from the unique *Kpn*I target within the 658bp *Xho*I fragment, also led to increased amidase expression. Together these findings suggest that *amiR* could also be transcribed from a promoter upstream of the 658bp *Xho*I fragment. Whether this alternative promoter is used *in vivo* is impossible to tell from these experiments; the increased expression could have been because a transcription terminator had been deleted in these constructs and *amiR* expression was now been driven from the *amiE* promoter.

Surprisingly plasmid pSW3, with a deletion of the 658bp *Xho*I fragment showed constitutive expression. This suggested that this DNA fragment either contained a *cis* acting sequence which regulated *amiR* expression or encoded a negative control element for amidase expression. The transcomplementation experiment with pDC5, pSW35, showed that inducer did not interact directly with AmiR, thus either *amiR* expression or the activity of the protein are regulated by some other factor which presumably responds to inducer.

To determine whether the DNA upstream of *amiR* might encode a repressor gene, two derivatives of the wild type inducible genes were made.

Firstly a 250bp *Cla*I-*Cla*I deletion was made which was downstream from the proposed promoter element within the 658bp *Xho*I fragment but upstream from the *amiR* coding sequence. Secondly an 8bp *Bam*HI linker was inserted into the unique *Eco*RV target within the 658bp *Xho*I fragment. This insertion was designed to cause a frameshift mutation in any potential regulatory gene. Both plasmids gave constitutive expression, thus providing the first evidence that there was an additional regulatory gene upstream of *amiR*.

The DNA sequence upstream of *amiR* was determined between *Xho*I(2400) and *Pvu*II(3700) and an open reading frame identified, corresponding to the regulator gene and named *amiC*. Homology searches with the *amiC* sequence and the EMBL sequence library failed to find any other proteins with homology. The 5' end of the *amiC* gene was only 136bp downstream from *Xho*I (2400) thus the *amiC/amiR* promoter element had to lie within this first 136bp of the 658bp *Xho*I fragment. Two DNA sequences were found which had homology with the *ntrA* dependent consensus promoter. A more detailed analysis of these promoters is covered in chapter 6. The *amiC* stop codon and the *amiR* start codon overlap so it seems likely that the two genes form part of an operon and are coordinately regulated.

One potential mechanism for AmiC action is as a conventional repressor functioning within an autogeneous control circuit whereby *amiC* regulates expression of itself and *amiR*. A promoter probe vector was constructed to look for promoter activity in the 658bp *Xho*I fragment and test the AmiC repressor model. However only one construct, pSW12 in *E.coli*, showed promoter activity with the 658bp *Xho*I fragment. Thus the role of the *ntrA* dependent promoters in the regulation of amidase expression was not established and the model for AmiC regulation was not tested. To investigate the mechanism of action of AmiC it became clear that purification of the protein for *in vitro* experiments e.g. DNA binding studies was going to be a useful exercise and the following chapter describes the attempts to over express and purify AmiC.

CHAPTER FOUR - OVER EXPRESSION, PURIFICATION AND N-TERMINAL SEQUENCING OF AmiC

4.1 Introduction

A thorough investigation of the mechanism of antitermination by AmiR and the mechanism of action of AmiC would be greatly facilitated by the purification of the proteins, which could then be used in *in vitro* experiments. However the purification of regulatory proteins is fraught with complications, the two major ones being i) they are often produced in very small quantities, ii) it is often difficult to set up simple *in vitro* assays to follow the purification. To over produce a regulatory gene generally requires the construction of an expression vector designed to maximise transcription and translation of the target gene. This is not a straightforward process since the over production of some regulatory proteins can have disastrous effects on the cell and cause loss of viability. Consequently most expression vectors used are designed to control the expression of the target genes. In this way a large biomass of cells can be grown before target gene expression is switched on, thus maximising production of the regulatory protein.

Initial attempts to identify and purify AmiR by analysis of the differences in elution profiles of dually labelled crude extracts isolated from *amiR*$^+$ and *amiR*$^-$ strains of *P.aeruginosa* separated on a variety of columns, were unsuccessful (Farin, 1976). This was at least partially due to the lack of a *P.aeruginosa* strain which over expressed *amiR* to a significant extent. Since the identification of the *amiR* open reading frame it has become possible to construct expression vectors to over produce AmiR. However, the only physical evidence of AmiR was obtained using an *in vitro* transcription/translation system with a variety of recombinant plasmids carrying the *amiR* gene (Cousens, 1987). This chapter describes attempts to over produce both AmiR and AmiC firstly using non-controllable expression vectors in *E.coli* and subsequently using controlled broad host range expression vectors in *E.coli* and

P.aeruginosa. In the case of AmiC the experiments were successful and resulted in the purification of the protein and the determination of the N-terminal amino acid sequence.

4.2 Over expression of *amiR* and *amiC* in *E.coli*

It was initially decided to try and over express both *amiC* and *amiR* using the small high copy number vectors pUC18 and pUC19 (Yanisch-Perron *et al*, 1985). These vectors have a *lac* promoter to drive expression of a foreign gene and have extremely high copy numbers, particularly when cultures go into stationary phase. Both vectors contain multiple cloning sites downstream of the *lac* promoter, providing convenient restriction targets for subcloning *amiR* and *amiC*. The major drawback with these vectors is that expression from the *lac* promoter is poorly controlled because insufficient *lac* repressor is produced by the host, and is titrated out by the large number of *lac* operators present with these high copy number vectors. Thus genes cloned downstream of the *lac* promoter are transcribed constitutively (Thompson, 1982), which could present problems if the foreign gene product is lethal to the cell. With this in mind the following two expression vectors were constructed.

4.2.1 Construction of plasmids pSW24 and pSW26

Plasmid pSW24 has the *amiR* gene subcloned into pUC19 and was constructed as follows: the 1.5kb *Xho*I fragment from pAS20 was subcloned into the *Sal*I target of pUC19. The orientation of the fragment with respect to the *lac* promoter was determined by restriction mapping with *Hin*dIII, *Cla*I and *Eco*RI. Plasmid pSW26 contains the 1.3kb *Kpn*I-*Pvu*II (*amiC*) fragment from pAS20 subcloned into the *Kpn*I and *Hinc*II targets of pUC18. The structure of the recombinant plasmid was confirmed by restriction mapping. Restriction maps of both plasmids are shown in Figure 4.1. pSW26 was very difficult to make and it took many attempts before this plasmid was isolated. One possibility was that *amiC* expression in *E.coli* at high levels was lethal and that this was the reason for the difficulty in constructing pSW26.

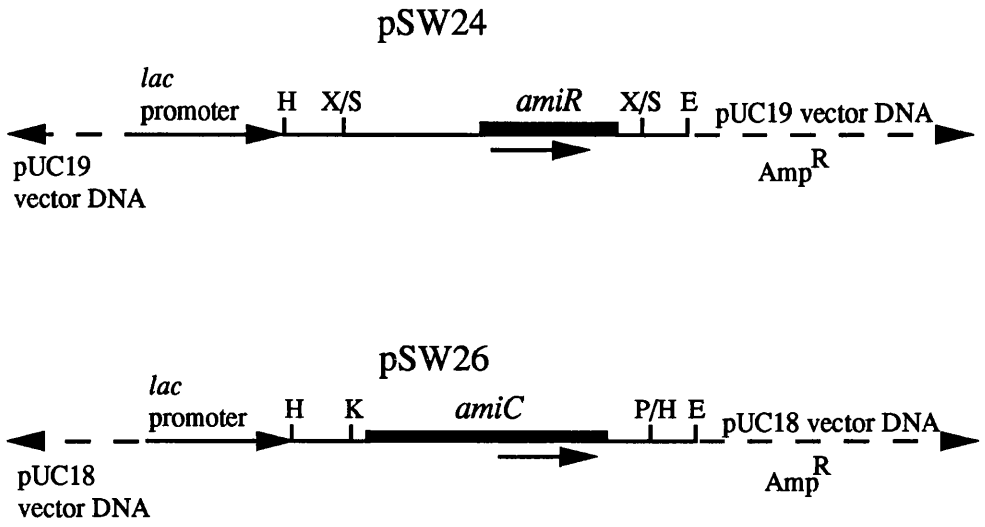

Figure 4.1. Restriction maps of plasmids pSW24 and pSW26. The maps are not drawn to scale. The arrows indicate the direction of transcription of *amiC* and *amiR* from the *lac* promoter. pSW24 consists of the 1.5kb *Xho* I fragment from pAS20 subcloned into the unique *Sal* I target in pUC19. pSW26 consists of the 1.3kb *Kpn* I - *Pvu* II fragment from pAS20 sucloned into the *Kpn* I and *Hinc* II targets of pUC18. The abbreviations for the restriction targets are as follows:- H= *Hind* III, K= *Kpn* I, E= *Eco* RI, X/S = the hybrid site generated by ligation of *Xho* I and *Sal* I restriction sites, P/H= the hybrid site produced by ligation of *Pvu* II and *Hinc* II restriction targets.

4.2.2 Analysis of crude extracts from strains containing pSW24 and pSW26.

To examine whether pSW24 and pSW26, over produced AmiR and AmiC respectively, crude extracts were prepared from the strains of *E.coli* JM101 bearing these plasmids and analysed using a 12% SDS-PAGE gel along with various control crude extracts. Analysis of JM101 containing pSW24 is shown in Figure 4.2. A comparison of the protein bands produced from JM101 containing pSW24 (+IPTG) (lane2) and JM101 containing pUC18 (+IPTG) (lane 5) shows no differences. The region of the gel which would be expected to contain AmiR (23kDa) is well resolved and there is no evidence of an extra band, corresponding to AmiR. The gel shown in Figure 4.2 also contains crude extracts prepared from JM101 containing pSW26 (lanes 3 &4). In both of these lanes there appears to be an extra band with an approximate molecular weight of 45kDa which could correspond to AmiC. These crude extracts were analysed further using an 8% SDS-PAGE gel.

The results of this analysis are shown in Figure 4.3. Lanes 6-10 contain various control crude extracts from JM101 and JM101, pUC18 grown in the presence of IPTG. Lanes which are described as containing pellets from crude extracts contain the cell debris produced from the clearing spin after sonication, which was subsequently resuspended in lysis buffer. Since the possibility existed that AmiC was a conventional repressor and pSW26 carries 135bp of DNA upstream of the gene itself, which could include an operator, crude extracts were prepared from JM101 containing pSW26 grown in the presence and absence of IPTG and in the presence of lactamide (inducer) or butyramide (repressor). In Lane 4 JM101 pSW26 grown in the absence of IPTG, no additional band corresponding to AmiC is visible. This result was surprising since it was expected IPTG would not significantly effect expression from the *lac* promoter in this construct. Lane 3 contains JM101 pSW26 grown in the presence of IPTG and contains a major new band presumably corresponding to AmiC with an approximate molecular weight of 46kDa. Lane 2 contains JM101 pSW26 grown in the presence of IPTG and lactamide and also

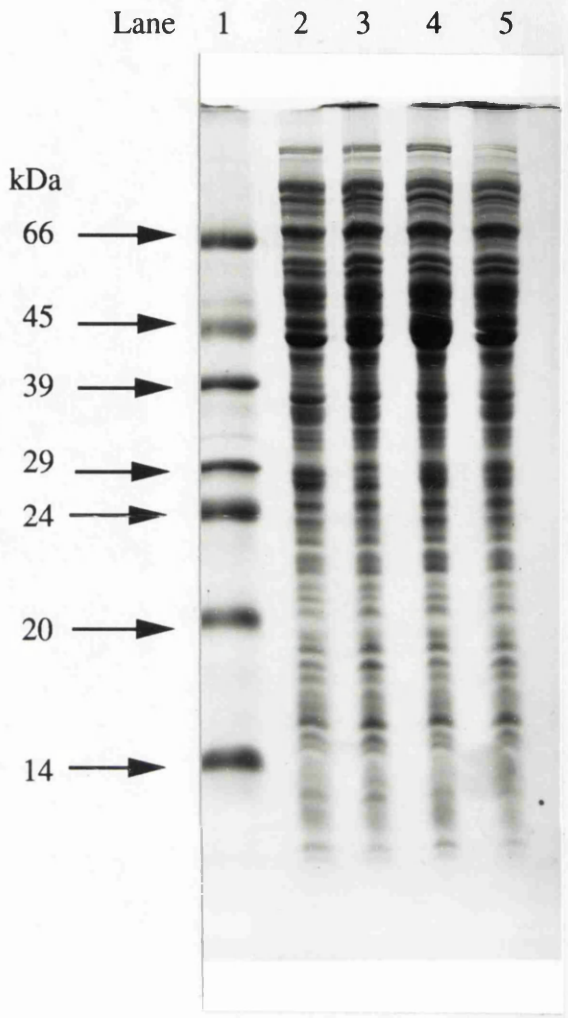

Figure 4.2. SDS-PAGE analysis of crude extracts from *E.coli* strain JM101 containing plasmids pSW24 and pSW26. Samples were run on a 12% SDS-PAGE gel. The sizes of the molecular weight standards are shown down the side of the photograph. Samples were loaded as follows:- lane 1, molecular weight standards; lane 2, crude extract of JM101 pSW24 + IPTG; lane 3, crude extract from JM101 pSW26 + IPTG + 0.2% lactamide; lane 4, crude extract of JM101 pSW26 + IPTG; lane 5, crude extract of JM101 pUC18 + IPTG.

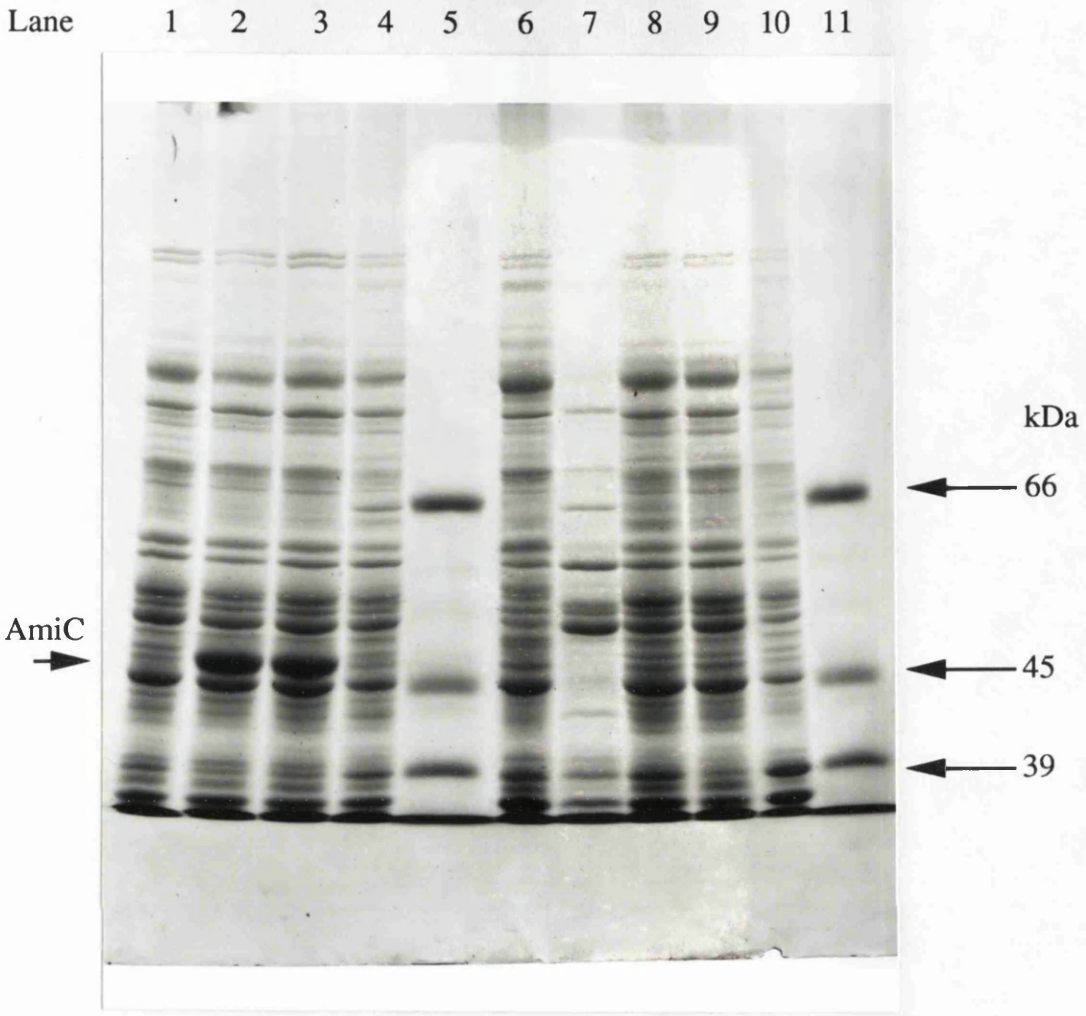

Figure 4.3. SDS- PAGE analysis of crude extracts from *E.coli* strain JM101 containing plasmid pSW26. Samples were run on an 8% SDS-PAGE gel. Molecular weight standard sizes are shown down the side of the photograph. Samples were loaded as follows: - lane 1, crude extract of JM101 pSW26 + IPTG + 0.2% butyramide; lane 2, crude extract of JM101 pSW26 + IPTG + 0.2% lactamide; lane 3, crude extract of JM101 pSW26 + IPTG; lane 4, crude extract of JM101 pSW26; lane 5, molecular weight standards; lane 6, crude extract of JM101 pUC18 + IPTG (pellet); lane 7, crude extract of JM101 pUC18 + IPTG; lane 8, identical to lane 6; lane 9, crude extract of JM101; lane 10, crude extract of JM101 (pellet); lane 11, molecular weight standards.

shows a major new band corresponding to AmiC. Lane 1 contains JM101 pSW26 grown in the presence of IPTG and butyramide and does not produce AmiC. Initially, it was thought that the absence of AmiC when cells were grown in the presence of butyramide might be because AmiC was repressing expression from the *lac* promoter by binding to an operator sequence immediately upstream of *amiC*. However, it was subsequently found that AmiC production from pSW26 was unstable since crude extracts did not consistently show evidence of the protein when analysed by SDS-PAGE (results not shown). This instability of AmiC production is probably the reason why no major new band is seen when JM101 pSW26 is grown in the absence of IPTG or in the presence of butyramide, in this particular gel.

The over production of foreign proteins in *E.coli* can be unstable particularly when the expression of the foreign gene is constitutive, as with pSW26. The problem of stability could be due to alterations in the plasmid structure during growth such as deletions, or rearrangements since JM101 is recombination proficient. Since the over production of AmiC appeared to lead to instability problems this would suggest its production was deleterious to the cell and might explain why pSW26 was such a difficult plasmid to isolate in the first place.

To overcome these problems it was decided to construct a broad host range expression vector where expression of *amiC* could be completely controlled and investigate *amiC* expression in *E.coli* and *P.aeruginosa*. The construction of plasmid pSW41 is described in Section 4.3. The inability to over express AmiR from pSW24 could have been for several reasons: firstly the *lac* promoter could have been too far away from *amiR* for efficient transcription, secondly *amiR* could be poorly translated in *E.coli* or thirdly AmiR might be an unstable protein.

4.2.3 Construction of plasmids pSW27 and pSW40

In an attempt to increase *amiR* expression from the *lac* promoter, a derivative of pSW24 was constructed whereby the *amiR* ORF was brought closer to the promoter by deletion of an intervening 250bp *Cla*I fragment. pSW24 was digested with *Cla*I, the 4kb fragment corresponding to pSW24 missing the 250bp *Cla*I fragment was isolated from an agarose gel and religated. The ligation mixture was transformed into *E.coli* JM101 and plasmid pSW27 isolated which has *amiR* 250bp closer to the *lac* promoter in pUC19. A restriction map of this plasmid is shown in Figure 4.4. The inability of plasmid pSW24 to over produce AmiR may have been a translational problem because of its highly biased codon usage (Lowe *et al*, 1989). Thus a broad host range *amiR* expression vector was constructed based on the expression vector pMMB66HE (Fürste *et al*, 1986). pMMB66HE is based on the promiscuous plasmid RSF1010 which can replicate in *E.coli* and *P.aeruginosa*. It contains the *lacI* gene, the hybrid *tac* promoter upstream of a multiple cloning site and the *E.coli rrnB* transcription terminator downstream of the multiple cloning site, which helps to stablise the vector-host system by preventing over expression of plasmid encoded genes from the *tac* promoter.

The broad host range *amiR* expression vector (pSW40) was constructed as follows: the 1.5kb *Hin*dIII-*Eco*RI fragment from pSW24 was subcloned into pMMB66HE cut with *Hin*dIII and *Eco*RI. The structure of the recombinant plasmid was confirmed by restriction mapping with *Hin*dIII, *Pvu*II and *Eco*RI. A restriction map of plasmid pSW40 is shown in Figure 4.4. Plasmid pSW40 was mobilised into the Ami deletion strain PAC452, by a triparental mating using the helper plasmid pRK2013.

Strain PAC452 is thought to have a chromosomal deletion encompassing the amidase locus since neither *amiE* or *amiR* can be complemented with recombinant plasmids to give amidase expression (R.Drew, unpublished results).

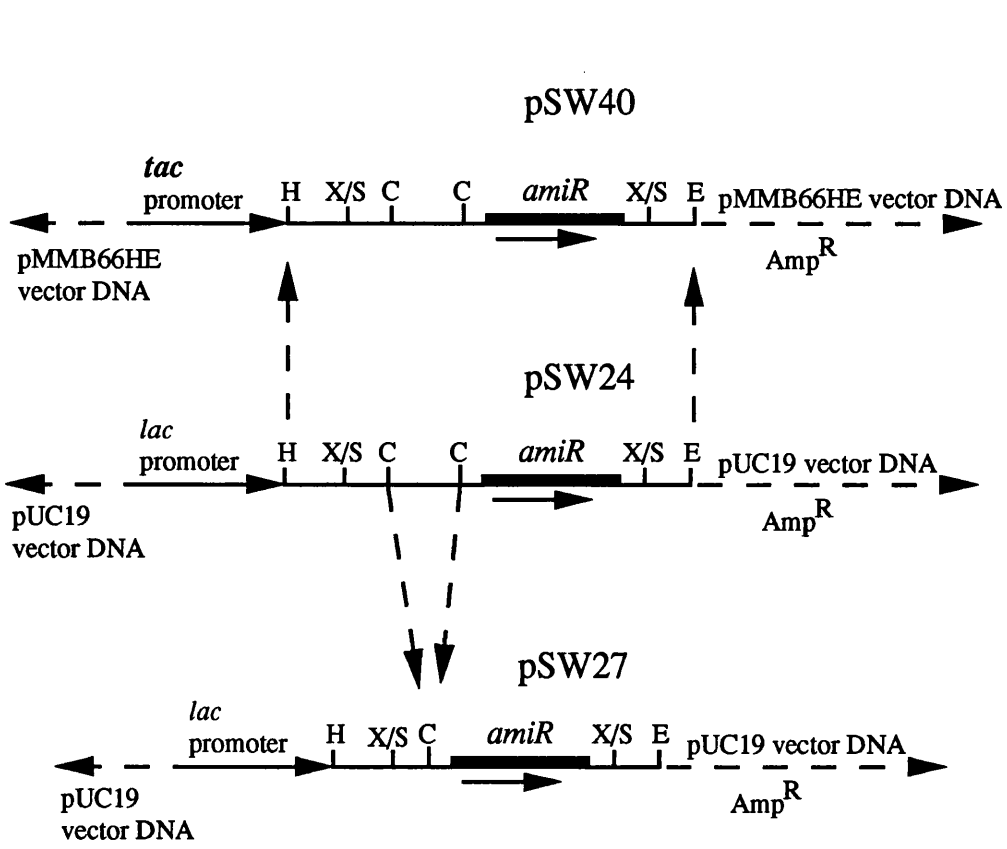

Figure 4.4. The construction of plasmids pSW40 and pSW27 from plasmid pSW24 and their restriction maps. The maps are not drawn to scale. The arrows indicate the direction of transcription *amiR* from the *lac* or *tac* promoter.The abbreviations for restriction targets are as follows:- H= *Hind* III, K=*Kpn* I, E= *Eco* RI, X/S = the hybrid site generated by ligation of *Xho* I and *Sal* I restriction sites, C= *Cla* I. pSW27 has the 250bp *Cla* I target from pSW24 deleted. pSW40 contains the 1.5kb *Hind* III- *Eco* RI from pSW24 subcloned into the *Hind* III and *Eco* RI targets of pMMB66HE.

4.2.4 Analysis of crude extracts from pSW27 and pSW40 in *E.coli* and *P.aeruginosa*

Crude extracts were prepared from *E.coli* JA221 pSW27, JA221 pSW40 and *P.aeruginosa* PAC452 pSW40. Various control crude extracts were also prepared from these host strains bearing the parental expression vectors. The samples were analysed on a 15% SDS-PAGE and the results are shown in Figure 4.5. None of the crude extracts prepared from the *amiR* expression vectors show the production of a major new protein which might correspond to AmiR. It is unlikely that this is due to poor transcription of *amiR* since the *tac* promoter and the *lac* promoter are powerful promoters which have been used to over express a wide variety of proteins. The possibility that *amiR* is poorly translated could still apply even in a *Pseudomonas* host, since the *amiR* ribosome binding site has several mismatches with the 3' end of the 16s rRNA of both *E.coli* and *P.aeruginosa*. Consequently, translational initiation could be the rate limiting step preventing its over production. This problem could only really be overcome by altering the sequence of the ribosome binding site (RBS) or by providing an alternative RBS for *amiR*. Alternatively, AmiR might be a very unstable protein and be rapidly degraded in the cell.

Finally there is no simple *in vitro* assay for *amiR* at present it is very difficult to assess whether small amounts of AmiR are being produced by these expression vectors. Analysis of crude cell extracts is rather an insensitive method for detection of AmiR and until a suitable assay has been devised for AmiR it would seem difficult to attempt a purification. If AmiR is a very unstable protein then even with a suitable detection system it might be very difficult to isolate large quantities of pure protein. An analysis of these *amiR* expression vectors in transcomplementation assays with *amiE in vivo* is described in Chapter five.

4.3 Construction of plasmids pSW41 and pSW42

Since constitutive production of AmiC from pSW26 in *E.coli* appeared to lead to instability problems a broad host range controllable expression vector

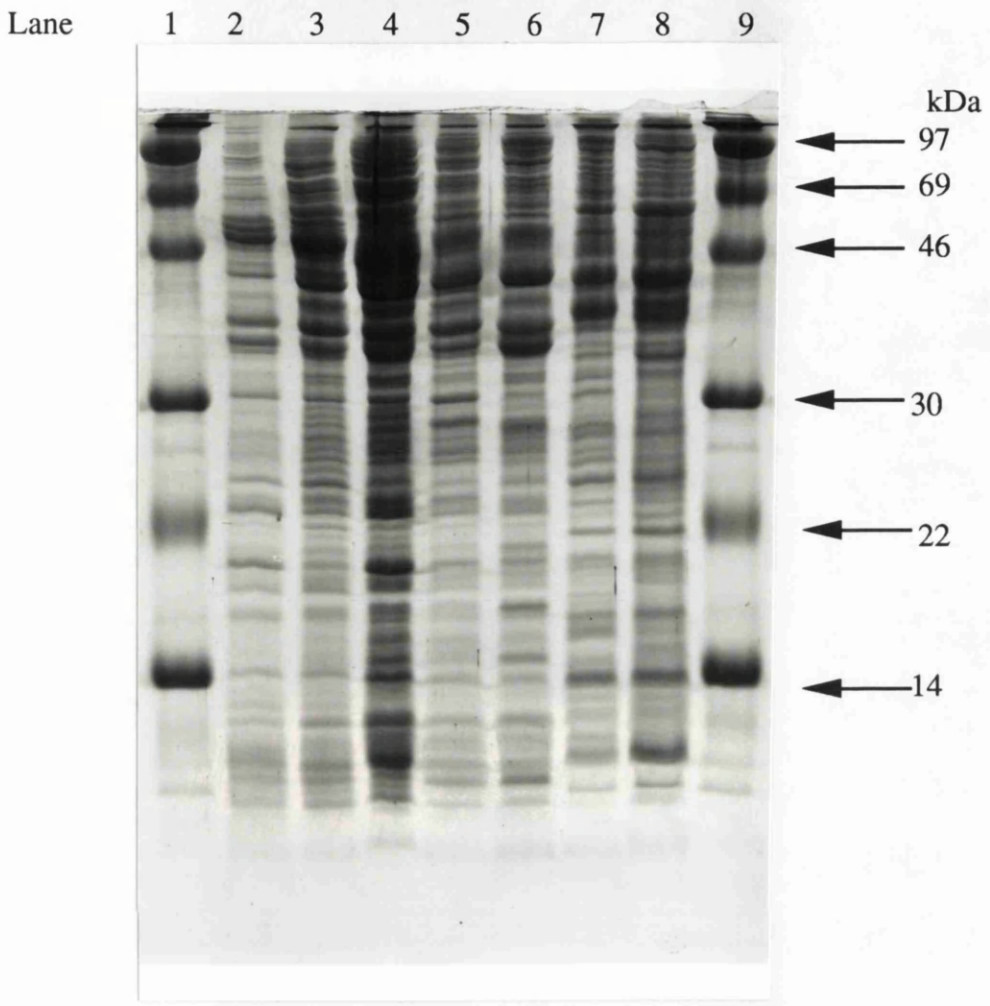

Figure 4.5. SDS- PAGE analysis of crude extracts from *E.coli* containing plasmids pSW27 and pSW40. Samples were run on an 15% SDS-PAGE gel. Molecular weight standard sizes are shown down the side of the photograph. Samples were loaded as follows: - lane 1, molecular weight standards; lane 2, crude extract of JM101 pUC18 + IPTG; lane 3, crude extract of JM101 pSW24 + IPTG; lane 4, crude extract of JM101 pSW27; lane 5, crude extract of JM101 pSW27 + IPTG; lane 6, crude extract of JA221 pMMB66HE + IPTG; lane 7, crude extract of JA221 pSW40; lane 8, crude extract of JA221 pSW40 + IPTG; lane 9, molecular weight standards.

based on the plasmid pMMB66HE described above, was constructed. Attempts to directly subclone the 1.4kb *amiC* *Hin*dIII-*Eco*RI fragment from pSW26 into the *Hin*dIII and *Eco*RI sites of pMMB66HE proved unsuccessful, since it was not possible to cleave the *Eco*RI target in pSW26. This suggested a mutation had occurred in this plasmid. Consequently the *amiC* gene had to be subcloned directly from pAS20 by digestion with *Kpn*I and *Pvu*II. This digestion releases two fragments with *Kpn*I and *Pvu*II ends which are of very similar sizes (1.4kb) and are impossible to separate on an agarose gel. The two fragments are *Pvu*II(1000)-*Kpn*I(2400) which contains the 3' end of *amiE* and the remaining DNA between *amiE* and *amiC* and the second fragment contains *amiC* (*Kpn*I(2400)- *Pvu*II(3780). These fragments were copurified on an agarose gel and the *Kpn*I cohesive ends were made blunt ended with T4 DNA polymerase. The fragments were then subcloned into pMMB66HE cut with *Sma*I and two new plasmids identified by restriction mapping with *Hin*dIII and *Xho*I. Plasmid pSW41 contains *amiC* in the correct orientation for expression and pSW42 contains the DNA sequence between *amiE* and *amiC*.

Previous studies had shown that phage insertions in the sequence between *amiE* and *amiC* rendered *P.aeruginosa* strain PAO1 amidase negative (Rehmat and Shapiro, 1983). However the strains were leaky mutants and showed some growth an acetamide plates. These results suggested that this region of DNA might code for a further gene involved in amidase regulation. Since pSW42 contained the DNA between *amiE* and *amiC* it had the potential to over express any gene within this region. Restriction maps of both these plasmids are shown in Figure 4.6

4.3.1 Analysis of crude extracts of *P.aeruginosa* containing plasmids pSW41 and pSW42.

Crude extracts were prepared from *E.coli* JA221 pSW41 and JA221 pSW42. Plasmids pSW41 and pSW42 were also mobilised into *P.aeruginosa* strain PAC452 using pRK2013 in triparental matings and crude extracts were prepared from the *Pseudomonas* strains. All extracts were analysed on a 10% SDS-PAGE gel and the results are shown in Figure 4.7.

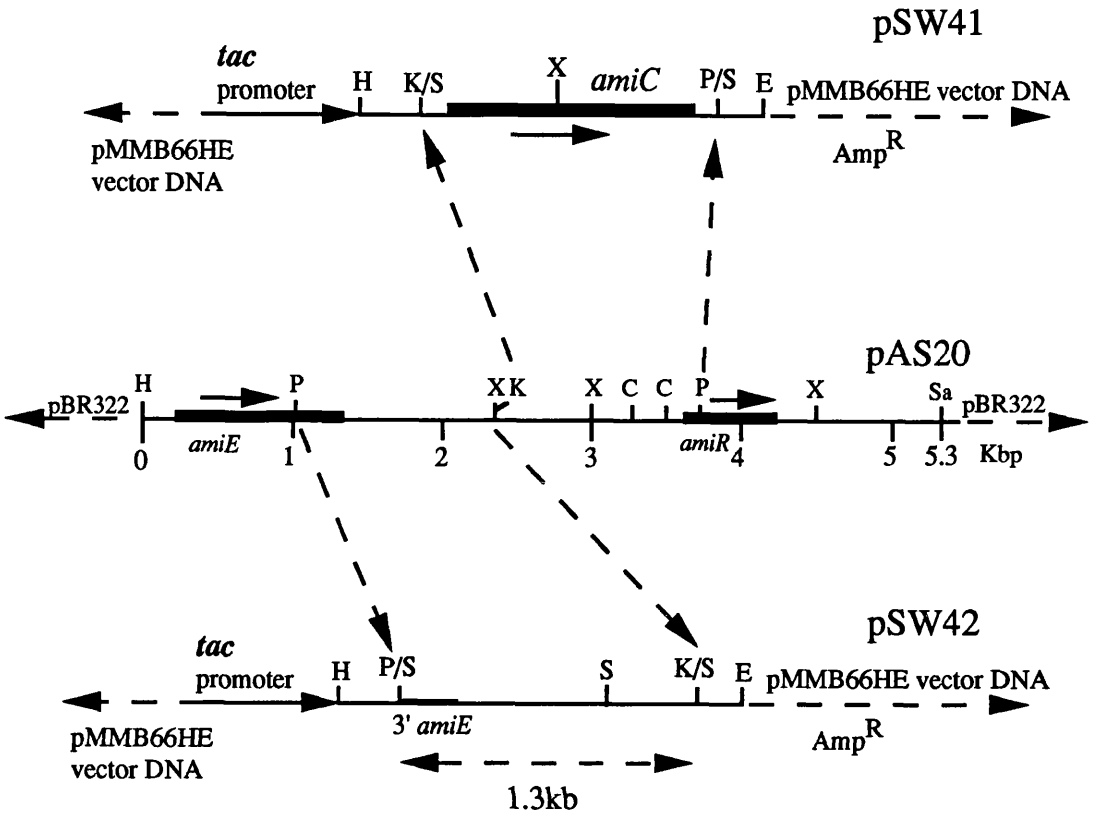

Figure 4.6. Restriction maps of plasmids pSW41 and pSW42. pSW41 consists of the 1.3kb *Kpn* I- *Pvu* II fragment from pAS20 subcloned into the *Sma* I target of pMMB66HE. pSW42 consists of the 1.3kb *Pvu* II- *Kpn* I fragment from pAS20 subcloned into the *Sma* I target of pMMB66HE. The abbreviations for the restriction targets are as follows:- H= *Hind* III, E= *Eco* RI, K/S = the hybrid site generated by ligation of a blunt ended *Kpn* I target and a *Pvu* II restriction site, S= *Sma* I, X= *Xho* I, P/S = the hybrid site generated by ligation of a *Pvu* II site with a *Sma* I site, C= *Cla* I, P= *Pvu* II, Sa= *Sal* I, K= *Kpn* I. The restriction maps are not drawn to scale.

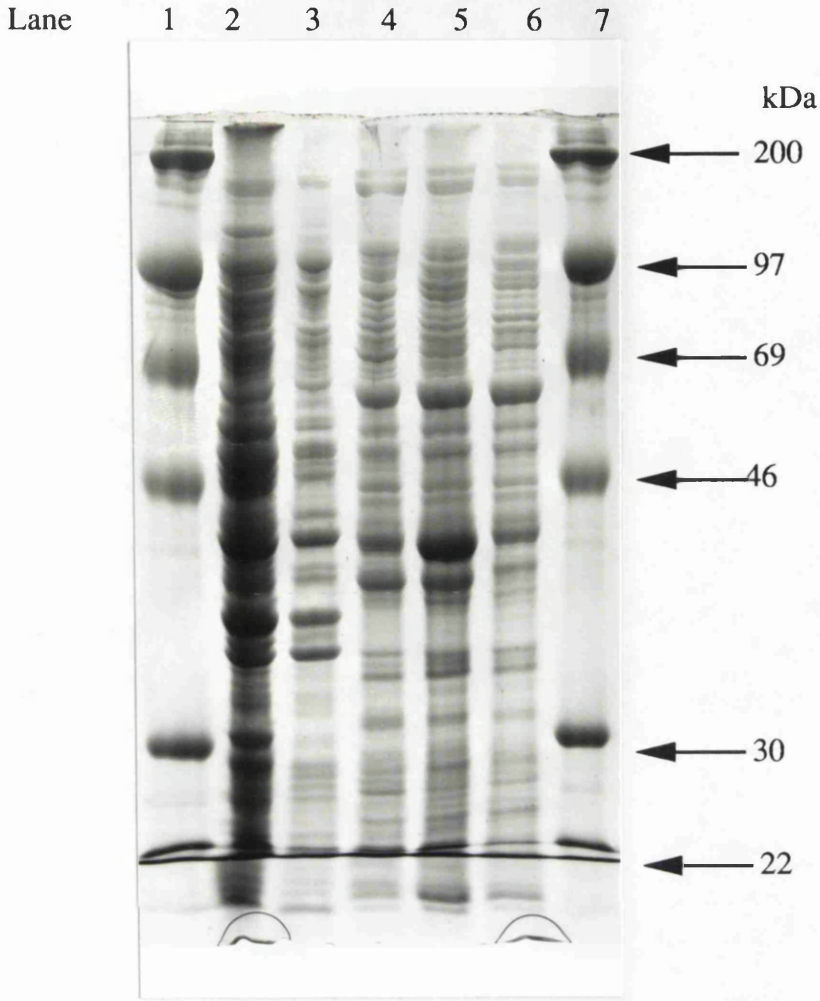

Figure 4.7 SDS- PAGE analysis of crude extracts from *E.coli* and *P.aeruginosa* containing plasmids pSW41 and pSW42. Samples were run on a 10% SDS-PAGE gel. Molecular weight standard sizes are shown down the side of the photograph. Samples were loaded as follows: - lane 1, molecular weight standards; lane 2, crude extract of JA221 pMMB66HE + IPTG; lane 3, crude extract of JA221 pSW41 + IPTG; lane 4, crude extract of PAC452 pMMB66HE + IPTG; lane 5, crude extract of PAC452 pSW41+ IPTG; lane 6, crude extract of PAC452 pSW42+ IPTG; lane 7, molecular weight standards.

E.coli JA221 pSW41 grown in the presence of IPTG (lane 3) does not appear to produce a new band corresponding to AmiC when compared to the control extract (*E.coli* JA221 pMMB66HE + IPTG) (lane 2). This was suprising, but the lack of AmiC production could be due to the low copy number of pSW41 (13).

The extract of PAC452 pSW41 shows a major new protein band (lane 5) compared with the control crude extract (lane 4). This protein has a predicted molecular weight of 43kDa which agrees closely with the molecular weight for AmiC, as predicted from the DNA sequence (42.8kDa). Plasmid pSW42 in strain PAC452 (lane 6) does not appear to produce a new protein compared with the control extract and in fact at least one of the control bands appears to be missing (lane 4). Since the molecular weight of any gene product encoded in this region is unknown, it is very difficult to assess from this insensitive detection method whether plasmid pSW42 is expressing any gene encoded by this region of DNA.

The resolution of the protein bands around the molecular weight of AmiC was not particularly good in the gel shown in Figure 4.7, so an 8% SDS-PAGE gel was run with PAC452 pSW41 crude extracts grown in the presence and absence of IPTG with control crude extracts and the results are shown in Figure 4.8.

Lanes 1 and 2 contain control crude extracts from PAC452 and PAC452 containing the parental expression vector pMMB66HE. Lane 3 contains an extract prepared from PAC452 pSW41 grown in the absence of inducer and surprisingly shows the production of a major new protein corresponding to AmiC. Lane 4 contains an extract prepared from PAC452 pSW41 grown in the presence of IPTG and shows greater over production of AmiC compared with lane 3.

The production of AmiC from PAC452 pSW41 in the absence of inducer was not expected since the *tac* promoter in this construct is supposed to be tightly regulated by the *lacI* gene on the plasmid and should only function in

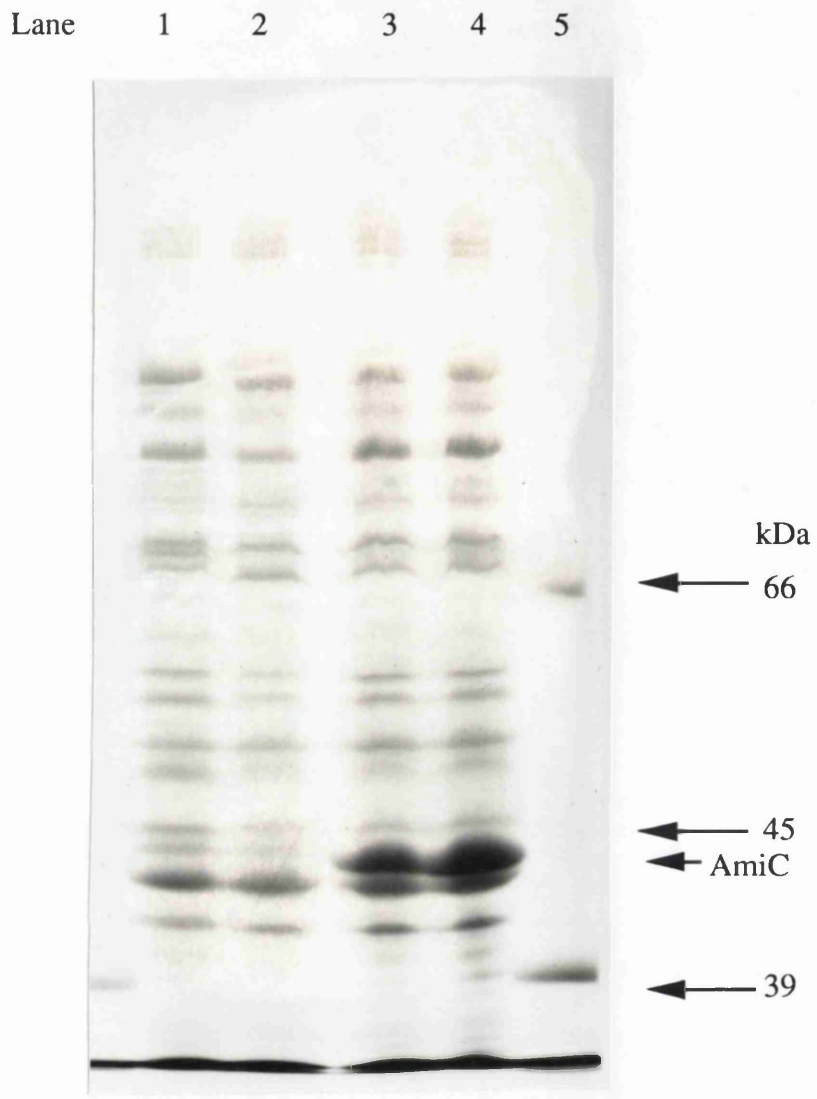

Figure 4.8. SDS- PAGE analysis of crude extracts from *P.aeruginosa* containing plasmid pSW41. Samples were run on an 8% SDS-PAGE gel. Molecular weight standard sizes are shown down the side of the photograph. Samples were loaded as follows: - lane 1, crude extract from PAC452; lane 2, crude extract of PAC452 pMMB66HE + IPTG; lane 3, crude extract of PAC452 pSW41; lane 4, crude extract of PAC452 pSW41+ IPTG; lane 5, molecular weight standards.

the presence of inducer. There are four possibilities which could explain AmiC production in the absence of IPTG: firstly the two *ntrA* dependent promoters upstream of *amiC* which are present in this construct could be directing transcription of the *amiC* gene, although previous experiments described in Chapter three suggest that these two promoter elements are weak; secondly there is some evidence that the *tac* promoter is not completely repressed in this expression vector (see Chapter five); thirdly a combination of the two *ntrA* dependent promoters and the *tac* promoter could be causing significant transcription of the *amiC* gene; fourthly, there could have been a mutation in *laci* or the *lac* operator during the cloning procedures, causing constitutive expression.

Since strain PAC452 pSW41 can produce significant quantities of AmiC it was decided to attempt a purification of AmiC from this strain and this is described below.

4.4 Purification of AmiC

The goal in mind when attempting to over express AmiC was to try and purify the protein, in sufficient quantity to investigate its mechanism of action with *in vitro* experiments. A second target was to try and obtain some N-terminal sequence from AmiC to ensure the correct start methionine and ORF for AmiC had been identified by the DNA sequencing studies. To this end an expression system has now been constructed which produces AmiC as one of the major intracellular proteins. Unfortunately when developing a rationale for the purification of AmiC there was no convenient *in vitro* assay and the only information available was that it was a major protein produced in the cell and had a subunit molecular weight of 43kDa. Consequently it was decided to try and purify AmiC firstly by fractionating the crude extract on a gel filtration column which separates proteins on the basis of size and then examining the fractions produced by SDS-PAGE for the presence of AmiC.

4.4.1 Gel filtration of a crude extract containing AmiC.

A crude extract was prepared from *P.aeruginosa* strain PAC452 containing plasmid pSW41 grown in the presence of IPTG. Approximately 5mg of this

crude extract was loaded onto an FPLC gel filtration column as described in materials and methods. The elution of proteins from the column was monitored by measuring the absorbance at 280nm. The elution profile from the gel filtration column is shown in Figure 4.9. Each fraction corresponds to 1ml of eluant from the column. Several major peaks can be seen on this graph which might correspond to AmiC. All fractions were collected and stored at 4°C overnight prior to SDS-PAGE analysis.

4.4.2 Analysis of gel filtration fractions by SDS-PAGE

To identify which fractions contained AmiC, 60µl from fractions 6-15 were boiled with 60µl of 2X loading buffer for 5 minutes and run on a 10% SDS-PAGE gel. The results of this analysis are shown in Figure 4.10. Fractions 11 and 12 both contain a major protein which comigrates with AmiC in the crude extract of PAC452 pSW41 (lane 11). This would indicate that the second major peak produced from the gel filtration column corresponds to AmiC (see Figure 4.9). Therefore the gel filtration of the crude extract appears to have resulted in substantial purification of AmiC as this second peak is clearly separated from the other major peaks. As a second stage of purification it was decided to perform ion exchange chromatography (IEC) on the two gel filtration fractions which contained AmiC. The fractions were not pooled for IEC since they appeared to contain different contaminants.

4.4.3 Ion exchange chromatography of fractions containing AmiC

AmiC has a pI predicted from the amino acid sequence of 6.7. In general a protein is negatively charged at pH values less than the pI and positively charged at pH values greater than the pI. Consequently it was decided to separate the gel filtration fractions containing AmiC on an anion exchange column where the mobile phase had pH8.0 and AmiC should be negatively charged and should bind to the column. The proteins that bound to the column would then be eluted with a linear salt gradient. To do this a Mono-Q column (Pharmacia) was used which binds negatively charged proteins through

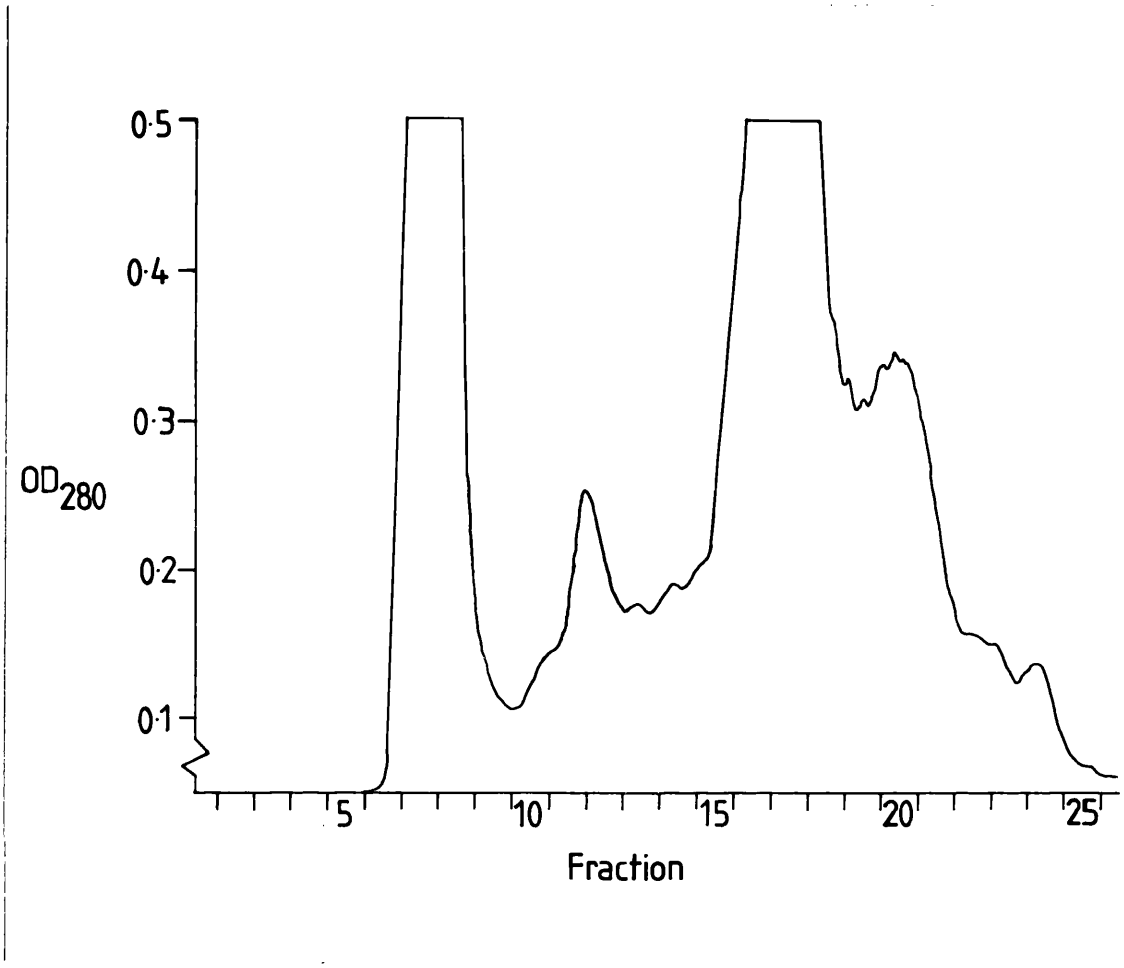

Figure 4.9. The elution profile from an FPLC gel filtration of a crude extract of PAC452 pSW41 grown in the presence of IPTG. Approximately 5mg of protein was loaded onto the gel filtration column. Each fraction corresponds to 1ml of eluant from the column.

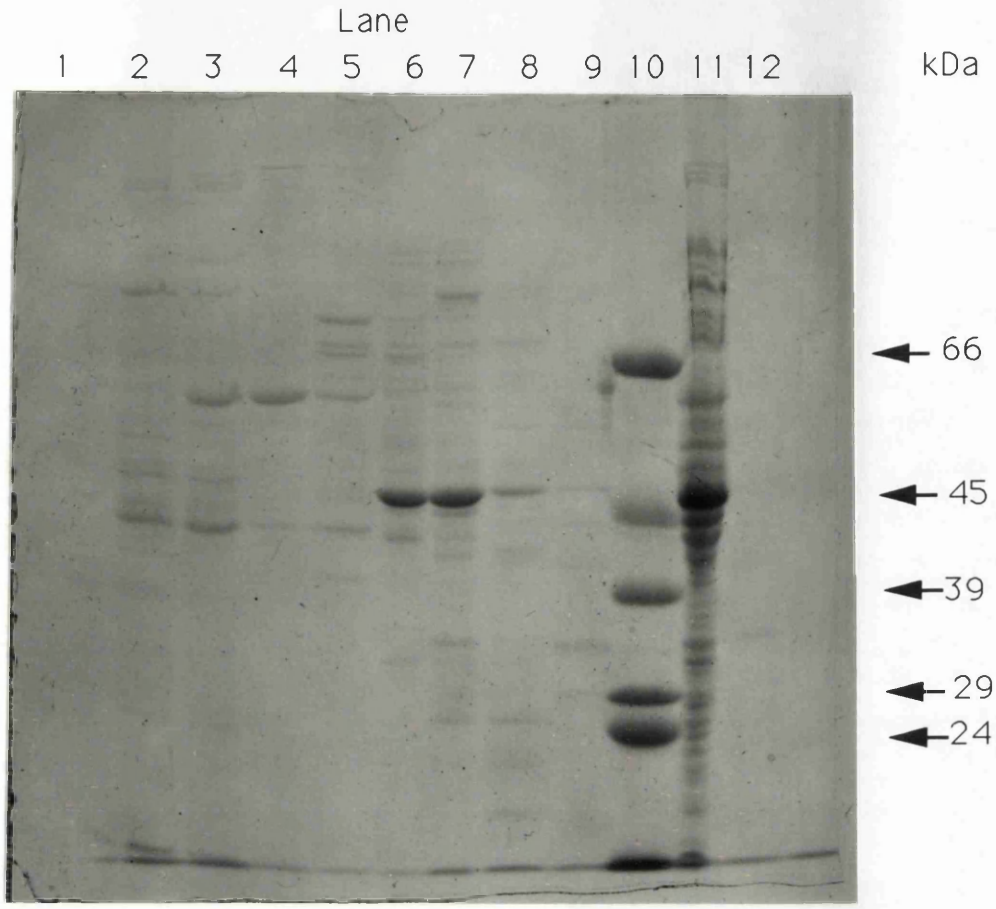

Figure 4.10. Analysis of gel filtration fractions by SDS-PAGE. Sample loadings were as follows: lane 1, fraction 6; lane 2, fraction 7; lane 3, fraction 8; lane 4, fraction 9; lane 5, fraction 10; lane 6, fraction 11; lane 7, fraction 12; lane 8, fraction 13, lane 9, fraction 14; lane 10, molecular weight marker; lane 11, crude extract of PAC452 pSW41; lane 12, fraction 15. AmiC is seen in fractions 11 and 12 with an approximate molecular weight of 45kDa.

quaternary amine groups which remain equally charged throughout the pH range 2-12. A linear salt gradient was used from 0-0.6M NaCl starting after 4ml of eluant had been collected and continuing until 24ml, then the gradient was increased from 0.6-1.0M to collect the final 5ml of eluant from the column. The elution profiles of the gel filtration fractions from the column are shown in Figures 4.11 and 4.12. The elution profile from fraction 11 shows four major peaks and the elution profile from fraction 12 shows a single major peak. The fractions were collected and analysed by SDS-PAGE as described below.

4.4.4 Analysis of ion exchange fractions by SDS-PAGE

Since gel filtration fraction 12 only yielded one major peak after ion exchange chromatography it was decided to perform SDS-PAGE analysis on the fractions collected around this peak. The analysis of these fractions from gel filtration fractions 11 and 12 are shown in Figures 4.13 and 4.14 respectively. With gel filtration fraction 11, all the fractions analysed appear to contain a band which comigrates with AmiC in the crude cell extract. However, fraction 14 appears to contain the most AmiC and in this fraction there is no evidence of other proteins. This fraction eluted at a salt concentration of 0.32M. This would indicate that the broad based peak eluting between 13 and 15 ml corresponds to AmiC. Gel filtration fraction 12 produces a single major peak with a small leading shoulder. The analysis of this peak shows that fractions 13-15 all contain a single protein band which comigrates with AmiC in the crude extract. Fraction 15 which elutes at a salt concentration of 0.32M appears to have the highest concentration of this protein, which is presumably AmiC.

The SDS-PAGE gels run here were stained with Coumassie Blue and the AmiC in fraction 15 appears to be the only protein band present, indicating that AmiC has been substantially purified. Fraction 15 produced by ion exchange of gel filtration Fraction 12 was subsequently used for N-terminal sequence analysis and this is described in section 4.5.

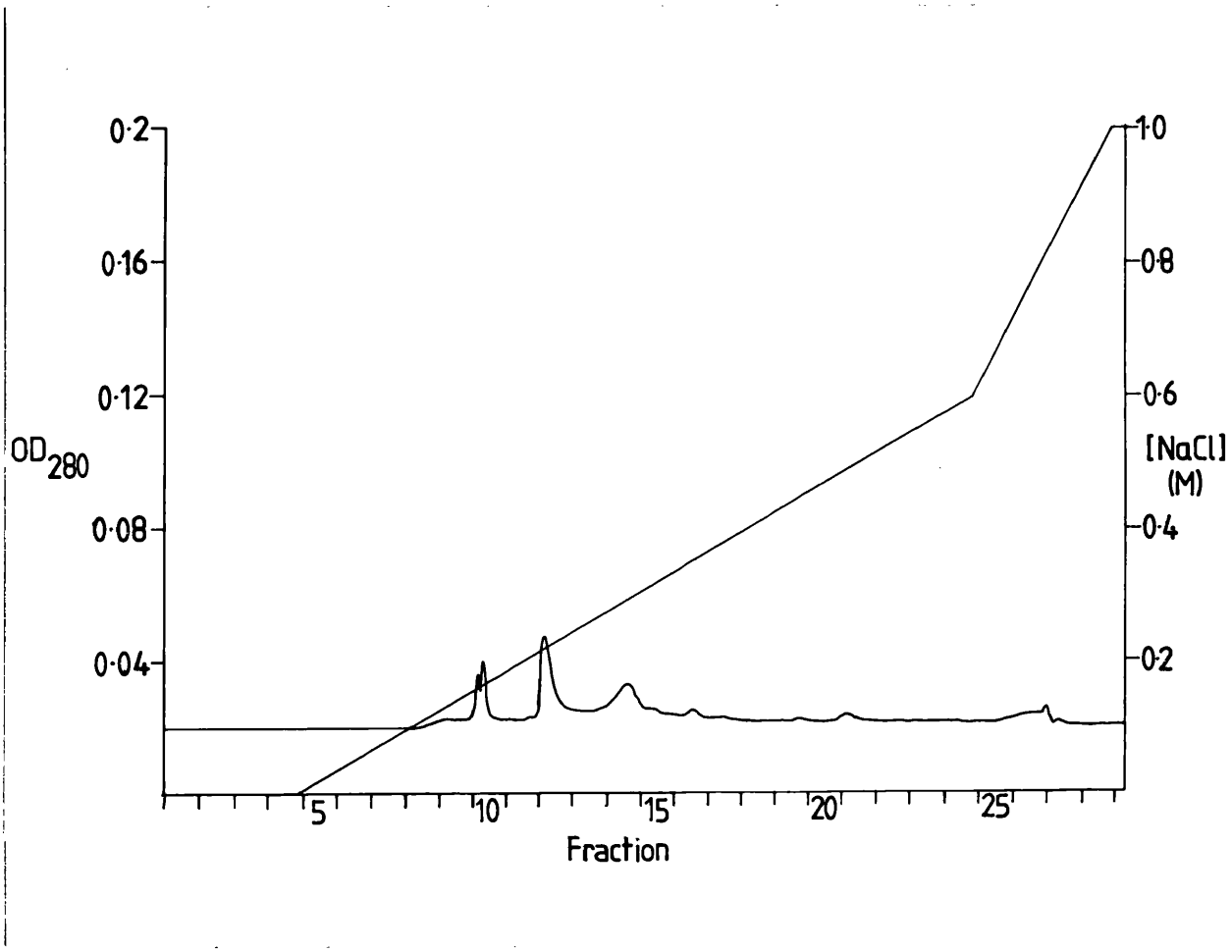

Figure 4.11. The elution profile from an FPLC Mono-Q ion exchange column, of gel filtration fraction 11 shown in Figure 4.9 and Figure 4.10. Each fraction corresponds to 1ml of eluant from the column. The straight line corresponds to the salt gradient used to elute the proteins from the column.

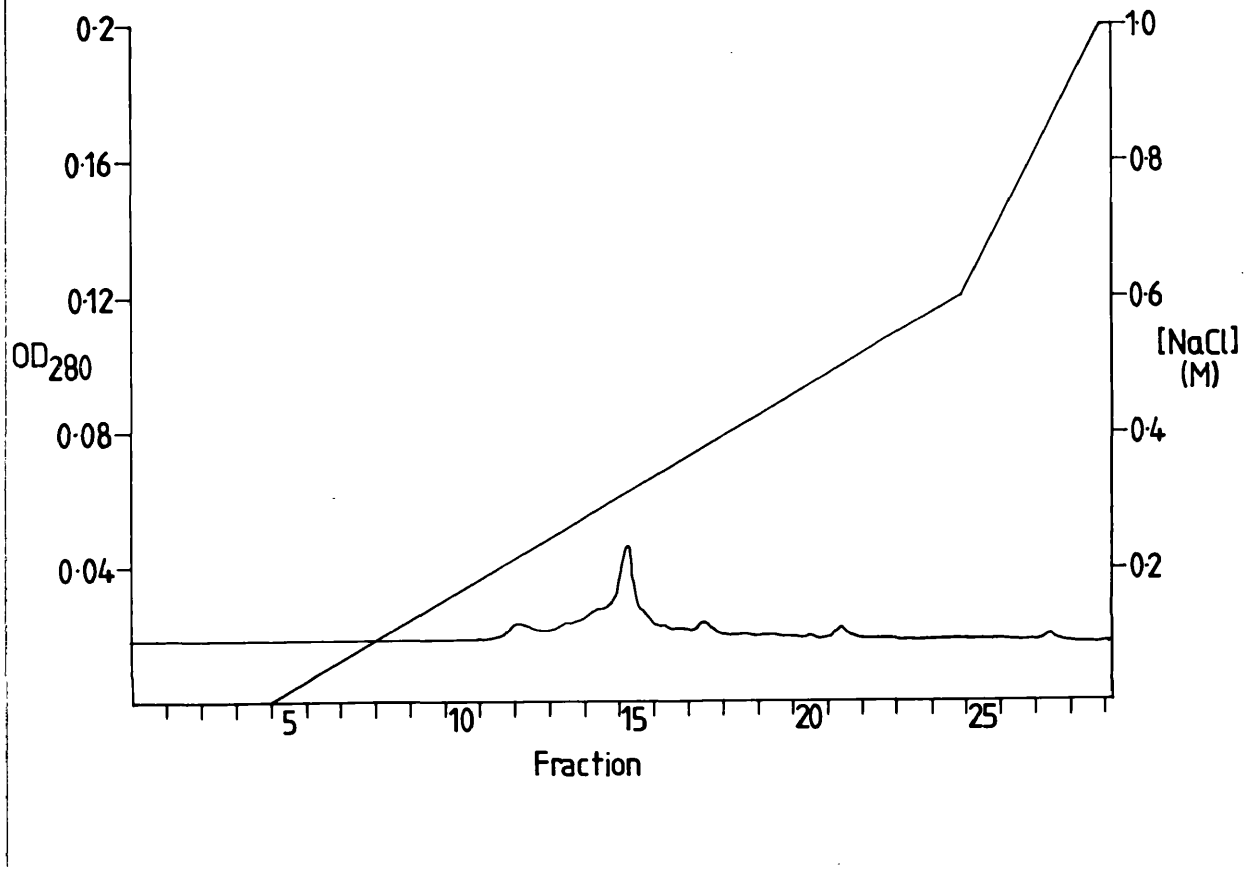

Figure 4.12. The elution profile from an FPLC Mono-Q ion exchange column, of gel filtration fraction 12 shown in Figure 4.9 and Figure 4.10. Each fraction corresponds to 1ml of eluant from the column. The straight line corresponds to the salt gradient used to elute the proteins from the column.

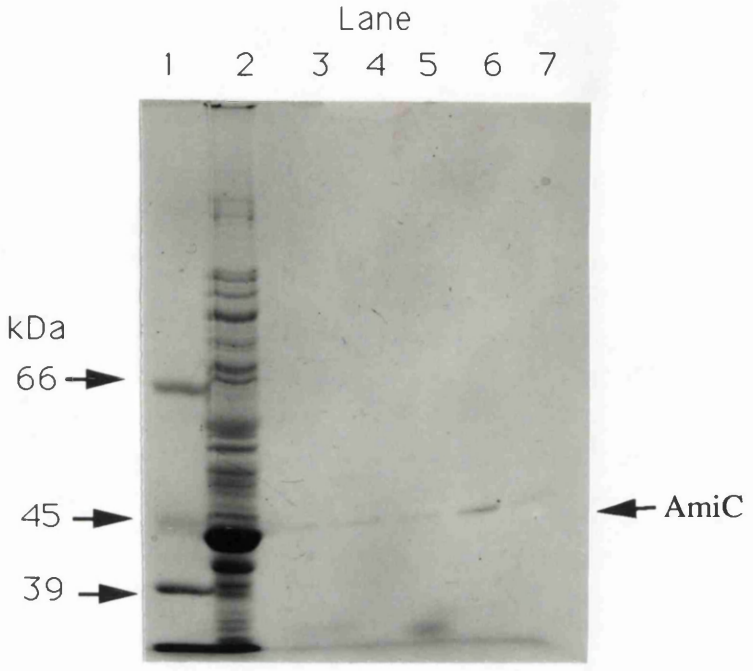

Figure 4.13 SDS-PAGE analysis of the fractions produced by ion exchange chromatography of gel filtration fraction 11. Sample loadings were as follows: lane 1, molecular weight markers; lane 2, crude extract of PAC452 pSW41; lane 3, fraction 11; lane 4, fraction 12; lane 5, fraction 13; lane 6, fraction 14; lane 7, fraction 15. AmiC is present in each fraction, though fraction 14 (lane 6) appears to show least contaminants.

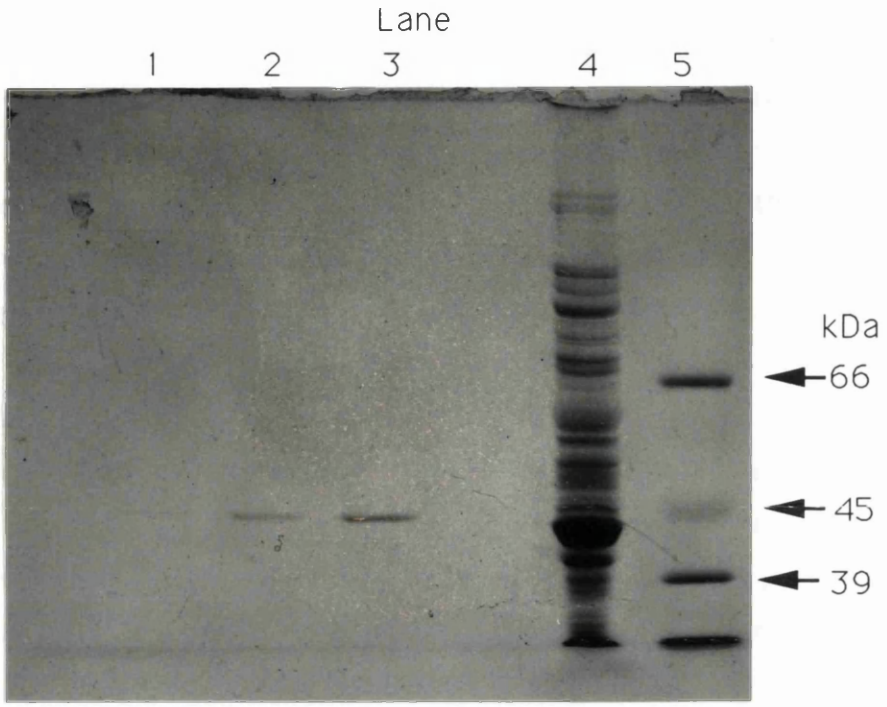

Figure 4.14 SDS-PAGE analysis of the fractions produced by ion exchange chromatography of gel filtration fraction 12. Sample loadings were as follows: lane 1, fraction 13; lane 2, fraction 14; lane 3, fraction 15; lane 4, crude extract of PAC452 pSW41; lane 5, molecular weight markers. AmiC is seen in fractions 14 and 15 (lanes 2+3). Fraction 15 was used for the N-terminal amino acid sequencing.

A worry with any protein purification procedure is its' reproducibility. Consequently fresh crude extracts were prepared of PAC452 pSW41 and subject to gel filtration as described above except that 10mg of protein was loaded on to the column. AmiC was found to elute at the same point as before. The two fractions containing AmiC were subsequently subject to ion exchange chromatography and the elution profiles from the column are shown in Figure 4.15 and 4.16. Substantially more protein was loaded onto the ion exchange columns this time and although AmiC eluted at 0.32M as shown previously as a large narrow peak, there were substantial contaminants (see fig 4.18). To try and remove some of these contaminants the AmiC containing fractions were pooled, from both ion exchange runs, desalted using a PD10 column (Pharmacia) and re-run on the ion exchange column. The salt gradient used for the elution was less severe than had been used previously, and it was hoped this might allow better separation of the contaminants from AmiC. The elution profile from the column is shown in Figure 4.17. The fractions were analysed by SDS-PAGE (see Figure 4.18) and it was found that AmiC eluted between 21-23 ml from the column at a salt concentration of 0.25M. The reason for the difference in the salt concentration at which AmiC eluted is unclear, however it could be because of the altered salt gradient. Fraction 22 contains a substantial amount of AmiC and appears to be free of other proteins. In conclusion it would appear that the AmiC purification protocol described above is generally reproducible however the resolution of proteins on the gel filtration column reduces when the loading capacity of the column is reached, as in the second gel filtration above and in this situation two ion exchange chromatography runs are needed to obtain pure AmiC.

4.5 N-terminal amino acid sequencing of AmiC

In prokaryotes the first amino acid of a protein is not methionine, but a derivative called *N*-formylmethionine (fMet). fMet is never retained as the N-terminal amino acid and has two possible fates. Either the formyl group is

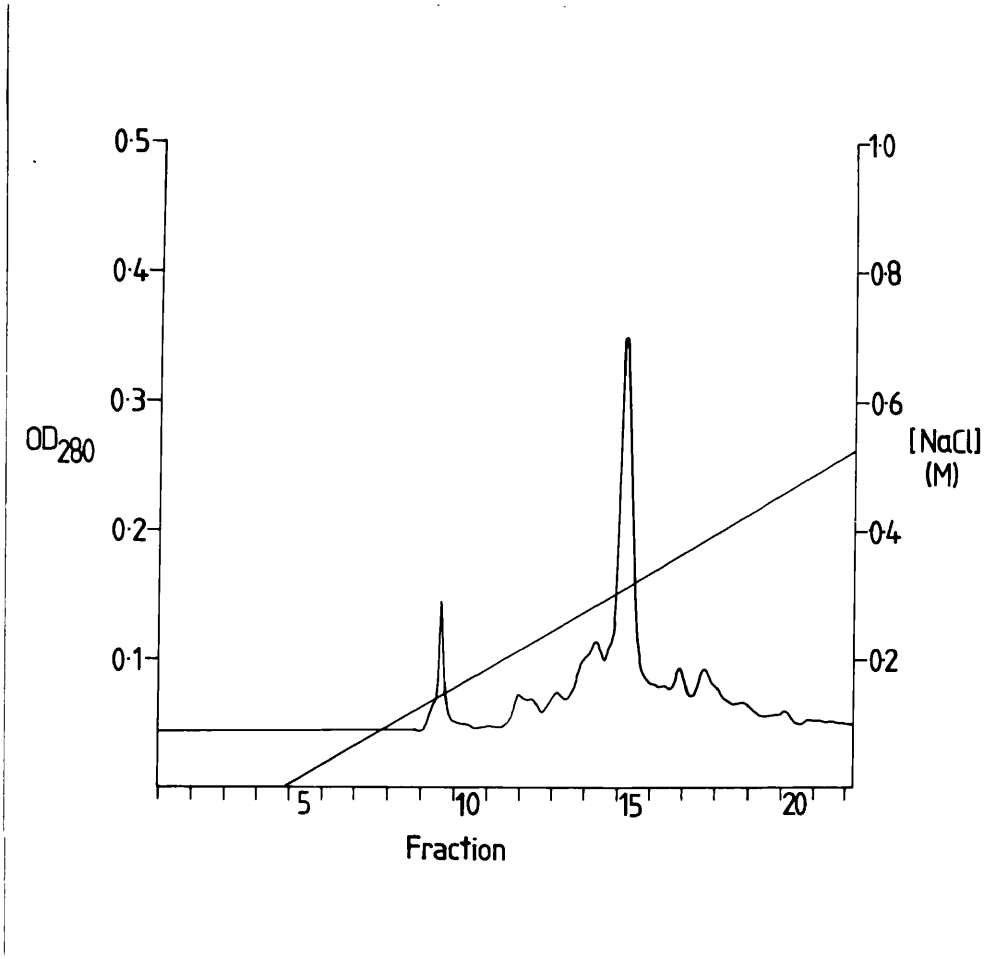

Figure 4.15. The elution profile from an FPLC Mono-Q ion exchange column of an AmiC containing gel filtration fraction . Each fraction corresponds to 1ml of eluant from the column. The straight line corresponds to the salt gradient used to elute the proteins from the column.

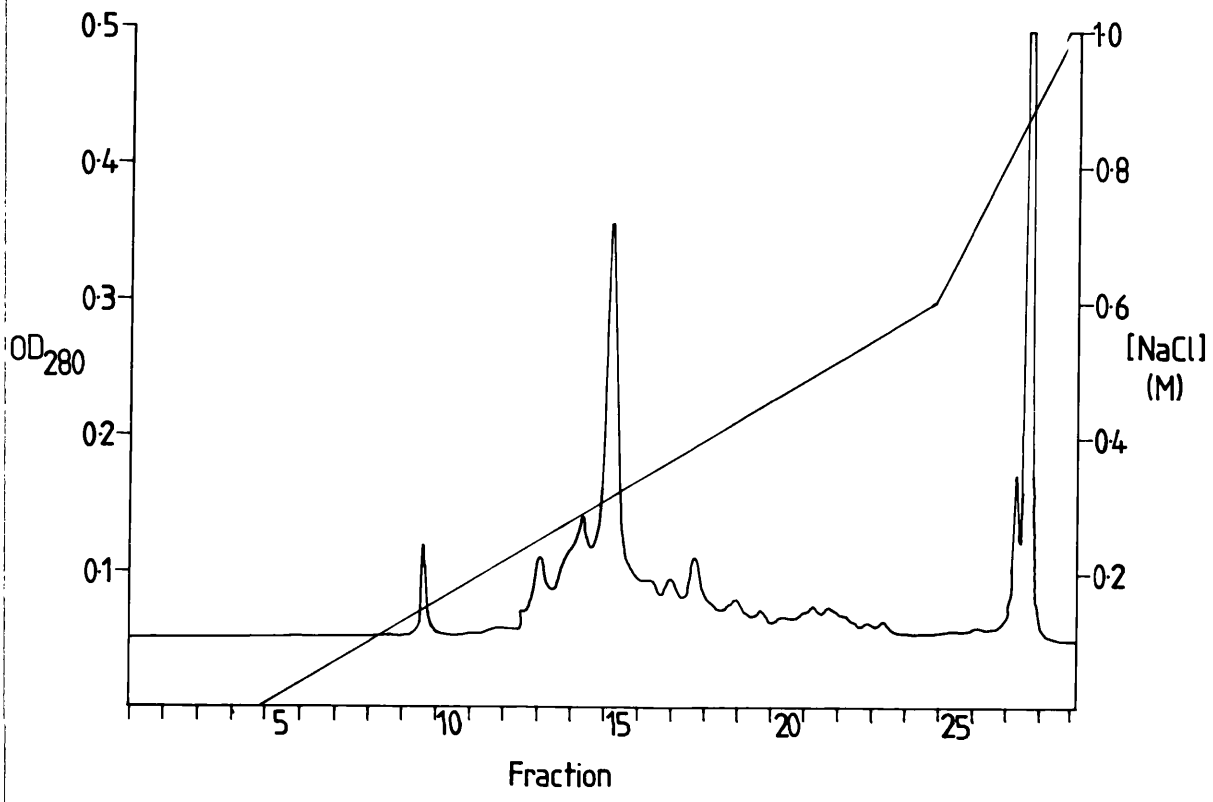

Figure 4.16. The elution profile from an FPLC Mono-Q ion exchange column of an AmiC containing gel filtration fraction . Each fraction corresponds to 1ml of eluant from the column. The straight line corresponds to the salt gradient used to elute the proteins from the column. AmiC corresponds to the large peak eluting between fractions 14 and 16.

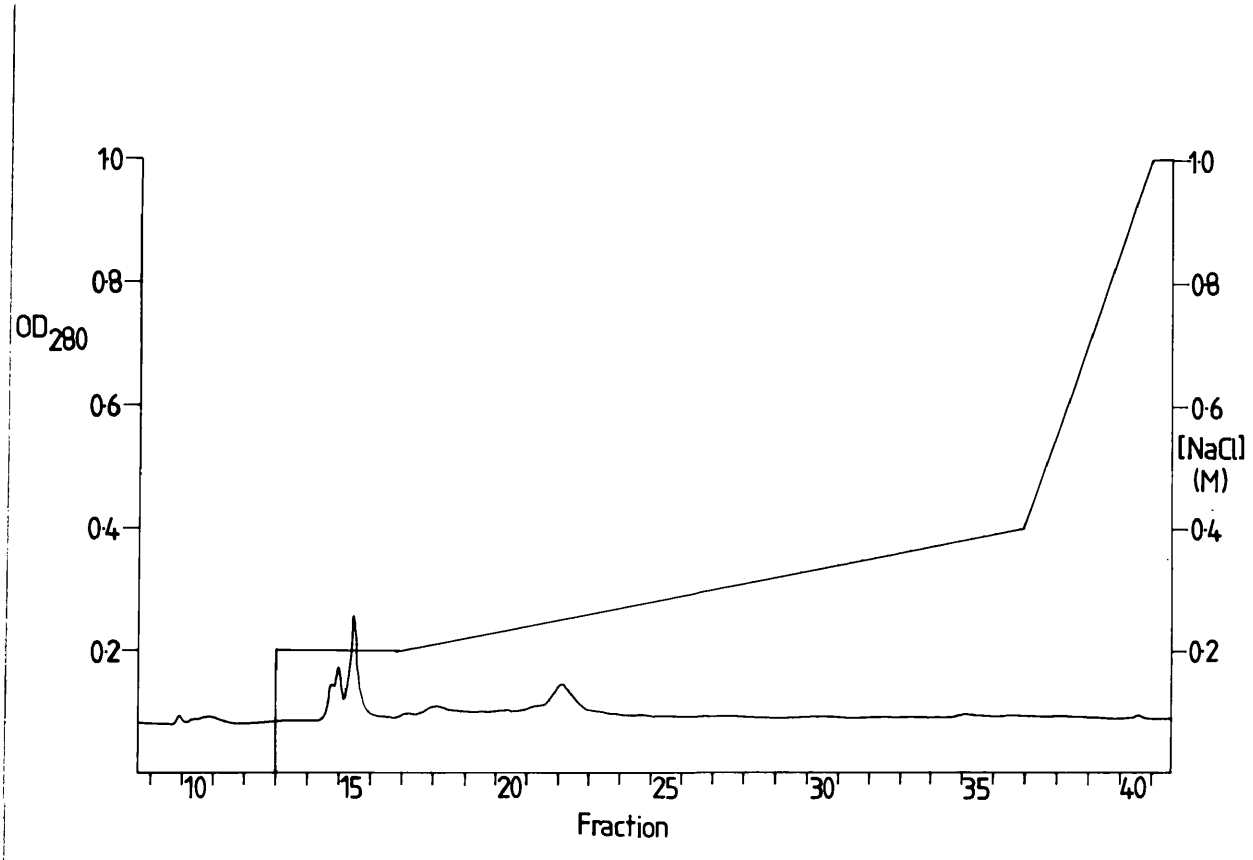

Figure 4.17. The elution profile from an FPLC Mono-Q ion exchange column of the AmiC containing ion exchange fractions shown in Figures 4.15 and 4.16. Each fraction corresponds to 1ml of eluant from the column. The straight line corresponds to the salt gradient used to elute the proteins from the column. AmiC corresponds to the peak eluting between fractions 21 and 23.

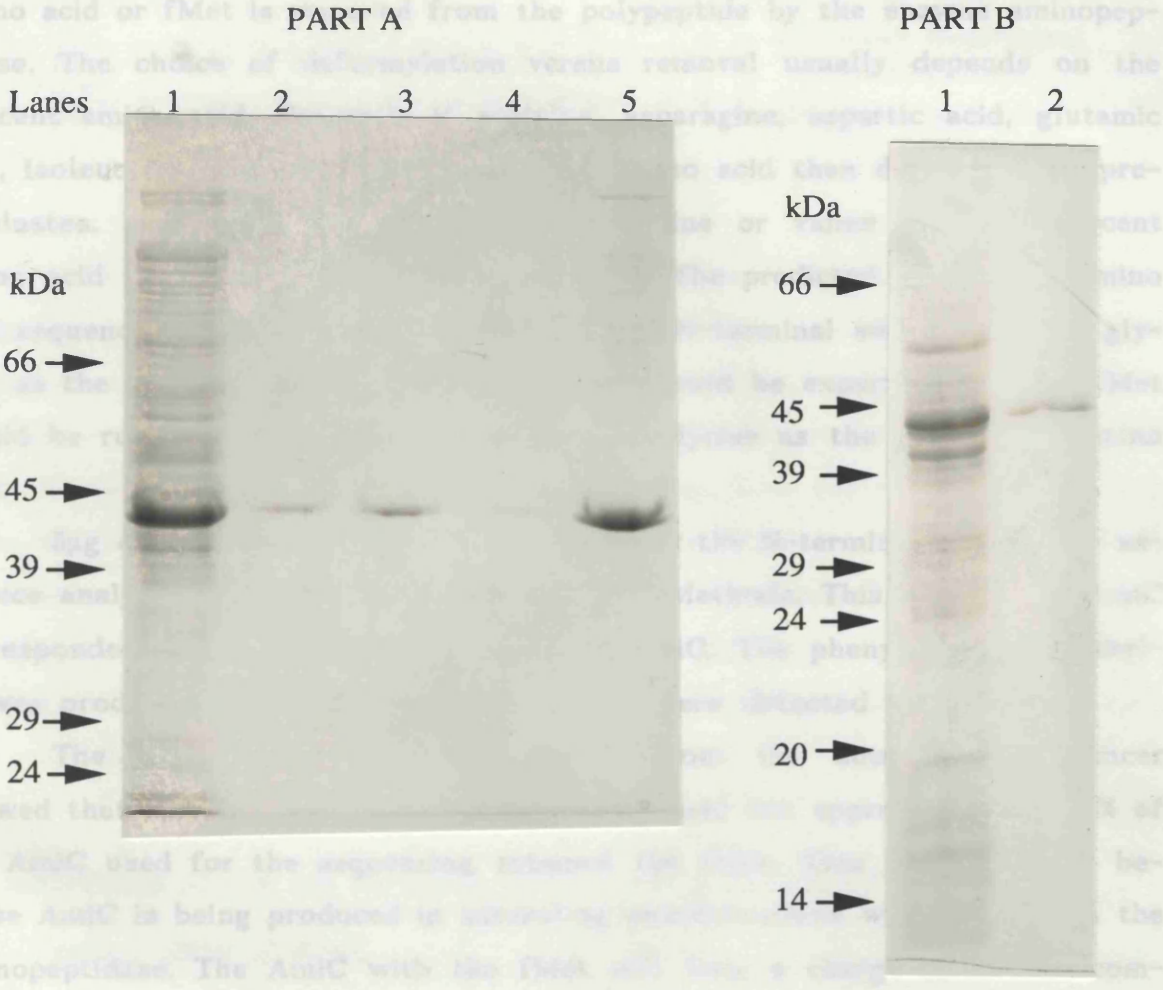

Figure 4.18 SDS-PAGE analysis of ion exchange fractions from the second attempt to purify AmiC. PART A shows samples analysed on a 12.5% SDS-PAGE gel. Samples were loaded as follows:- lane 1, crude extract of PAC452 pSW41 + IPTG; lane 2, fraction 21 from ion exchange column (see Fig. 4.17); lane 3, fraction 22 from ion exhange column; lane 4, fraction 23 from ion exchange column; lane 5, fraction 15 from the ion exchange run shown in Figure 4.16. PART B shows fraction 22 shown in PART A above run on a 15% SDS- PAGE gel. Sample loadings are as follows:- lane 1, crude extract of PAC452 pSW41 + IPTG; lane 2, fraction 22 from ion exchange chromatography.

removed by the enzyme deformylase, leaving methionine as the N-terminal amino acid or fMet is removed from the polypeptide by the enzyme aminopeptidase. The choice of deformylation versus removal usually depends on the adjacent amino acid. Generally if arginine, asparagine, aspartic acid, glutamic acid, isoleucine, or lysine are the adjacent amino acid then deformylation predominates. If alanine, glycine, proline, threonine or valine are the adjacent amino acid then the fMet is usually removed. The predicted N-terminal amino acid sequence for AmiC has methionine as the N-terminal amino acid and glycine as the adjacent amino acid, therefore it would be expected that the fMet would be removed by aminopeptidase to give glycine as the N-terminal amino acid.

5μg of FPLC purified AmiC was used for the N-terminal amino acid sequence analysis as described in Materials and Methods. This amount of AmiC corresponded to approximately 80pmols of AmiC. The phenylthiodantoin derivatives produced by the automatic sequencer were detected at 214nm.

The amino acid sequence obtained from the automated sequencer showed that glycine was the N-terminal amino acid but approximately 5-10% of the AmiC used for the sequencing retained the fMet. This is presumably because AmiC is being produced in saturating concentrations with respect to the aminopeptidase. The AmiC with the fMet will have a charge difference compared to the AmiC without fMet, which would account for the leading shoulder adjacent to the AmiC peak produced by the ion exchange column (Section 4.4.4). In fact the SDS-PAGE analysis of this leading shoulder only showed evidence of AmiC. A summary of the results from the N-terminal amino acid sequence analysis is shown in Figure 4.19.

M G S H Q E R P L I G L L F S E T G V T	Predicted N-terminal AmiC sequence
M* G S H Q E R P ? ? G L L F S ? T G V T	Sequence obtained

Figure 4.19. **Comparison of the N-terminal sequence obtained for with that predicted from the DNA sequence. "*" indicates that 5-10% of the sequenced AmiC retained the N-terminal methionine.**

There were injection problems with the sequencer for some of the amino acids and three amino acids were not clearly identified by this analysis. However seventeen of the first twenty amino acids were identified unambiguously. The sequence obtained matched the predicted amino acid sequence, thus confirming that the purified protein was AmiC and the correct start methionine and open reading frame had been identified.

4.6 Calibration of the gel filtration column and prediction of the native state of AmiC

Since gel filtration separates proteins on the basis of size it is possible to calibrate a gel filtration column with proteins of known molecular weight and subsequently calculate the molecular weight of an unknown. This method was used to assess whether AmiC existed *in vivo* as a monomer, dimer etc. The standards used for this analysis were split into two groups and run separately on the FPLC gel filtration column, allowing easy identification of the proteins as they eluted from the column. The two groups of standards used were as follows: i) Dextran Blue 2000 which is totally excluded from the column thus its elution volume represents the void volume of the column, catalase (Mwt = 232kDa), aldolase (Mwt = 158kDa) ii) bovine serum albumin

(Mwt = 67kDa), ovalbumin (Mwt = 43kDa), chymotrypsinogen A (Mwt = 25kDa). The elution of the standards was followed by monitoring absorbance at 280nm. To produce a calibration curve for the gel filtration the volume at which each protein eluted was recorded and a K_{AV} value was calculated as follows:

$$K_{AV} = \frac{V_e - V_O}{V_t - V_O}$$

where V_e = elution volume of the protein, V_O = column void volume = elution volume of Blue Dextran 2000 = 6.3ml, V_t = total column volume = 25ml.

The K_{AV} value of each protein was calculated and plotted against the log Mwt to produce the calibration curve shown in Figure 4.20

Unfortunately at the time of doing this analysis, insufficient purified AmiC was available to run on the gel filtration column to determine its molecular weight so the elution volume for AmiC from this column was calculated from the gel filtration elution profile for the crude extract of PAC452 pSW41. With this sample the apex of the peak corresponding to AmiC elutes at a volume of 11ml. This would give AmiC a K_{AV} of 0.25 which corresponds to a molecular weight of 128kDa on the standard curve. Since the subunit molecular weight of AmiC, calculated from the derived amino acid sequence is 42.8kDa this would indicate AmiC exists as a trimer. However, this analysis is far from satisfactory since the resolution of high molecular weight proteins on the FPLC column used is very poor and the presence of a vast number of other proteins in the crude cell extract might affect the elution of AmiC. These factors together amount to a considerable margin of error in this calculation, and one could only say with confidence that AmiC exists as at least a dimer and there is a possibility that the vast over expression of AmiC is leading to aggregation of the protein.

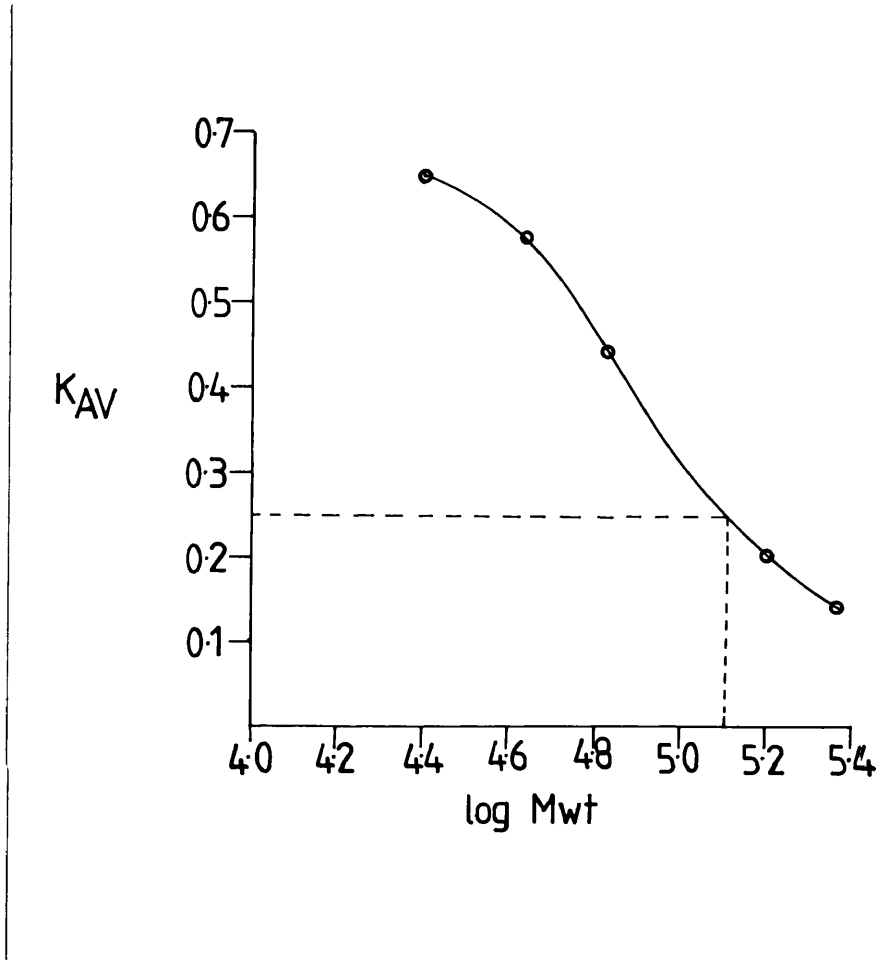

Figure 4.20. Gel filtration calibration curve for the FPLC gel filtration column used to purify AmiC. The derivation of KAV is shown in the text. The dotted line indicates the KAV and thus predicted molecular weight for the native form of AmiC.

4.7 Summary

The attempts to overexpress *amiR* in *E.coli* and *P.aeruginosa* described in this Chapter have been unsuccessful. There are several reasons which could account for this failure. Firstly, the ribosome binding site of *amiR* has several mismatches with the 3' end of the 16s RNA of both *E.coli* and *P.aeruginosa* (see Figure 4.21). It has been clearly shown that the rate limiting step in translation, is the initiation stage, and a large determinant in this initiation reaction is the quality and position of the ribosome binding site (McCarthy and Gualerzi, 1990). Therefore, despite maximising transcription of the *amiR* gene by placing it downstream of strong promoters (*lac* and *tac*), the mismatches in the RBS might prevent efficient translation of the mRNA. However, this explanation seems unlikely since the RBS binding site of *amiC* has a similar number of mismatches with the 3' end of the 16s rRNA and yet appears to be translated very efficiently. A second alternative, for the lack of a major new band on SDS-PAGE gels could be that the AmiR protein is unstable and is degraded during the cell harvesting and lysis procedures. The λ N antiterminator protein has a short half life and is degraded in the cell by the Lon protease (Maurizi, 1987). Consequently, there is the possibility that AmiR is degraded by a similar protease in *P.aeruginosa*.

5'----GGCGGGGGAGCGCUCCCAUG------- *amiR* leader sequence.

3'---- AUUCCUCCACUAG----------3' end of 16s RNA from *E.coli* and *P.aeruginosa* (sequences identical)

Figure 4.21. Comparison of the RBS binding site of *amiR* with the sequence of the 16s rRNA from *P.aeruginosa* and *E.coli*.

Since no major band corresponding to AmiR was identified by SDS-PAGE this automatically thwarted any attempt to purify this protein, since no other assay was available for the protein. To construct an assay system for AmiR based on the property of the protein to cause transcription antitermina-

tion, would have to be carried out using an *in vitro* transcription system. This would not be a trivial assay and is probably not the most sensible way to purify the protein. A simpler alternative would be to construct a fusion protein, with some protein that could be assayed. For example, vectors have now been designed where fusion proteins can be made between the maltose binding protein (*malE*) and the target gene (Guan *et al*, 1988). The fusion protein can then be isolated by affinity chromatography on an amylose column. The advantage of this system would be that native AmiR could be made since the hybrid protein can be designed so that it can be cleaved with Factor Xa at the fusion site to regenerate native maltose binding protein and native AmiR. Such a system would seem most attractive for AmiR purification since no direct assay for AmiR is required.

The over expression of AmiC got off to a bad start in *E.coli* since the expression vector pSW26 produced AmiC in a very unreliable manner. Identical growth conditions on separate occasions sometimes produced AmiC as a major new protein in the cell and other times produced nothing. This suggested that AmiC overproduction in *E.coli* might be toxic to the cell, which has been found for several foreign proteins expressed in *E.coli*. The constitutive expression of *amiC* in pSW26 probably contributed to the erratic AmiC production.

Plasmid pSW41 was constructed to provide controlled expression of *amiC*, and to allow over expression in the normal host. In *E.coli* this plasmid did not appear to over produce AmiC in large enough amounts to be visualised by SDS-PAGE. This could have been because of the highly biased codon usage of the gene (see Table 3.6). In *P.aeruginosa* the plasmid produced AmiC as one of the major intracellular proteins. The expression of *amiC* was surprisingly constitutive and yet appeared to be stable. This could be because the protein was being produced in *P.aeruginosa*, the natural host for AmiC.

Since no conventional assay was available for AmiC, the purification had to rely on the appearance of the protein on SDS-PAGE gels. This was quite satisfactory and resulted in substantial purification of the protein simply using

gel filtration and ion exchange chromatography. The AmiC protein was N-terminally sequenced to confirm that the correct protein had been purified and to ensure the correct start codon and reading frame had been identified by the DNA sequence analysis. The elution point of AmiC from the gel filtration column used indicated the protein existed as at least a dimer in its native state.

In the next Chapter the function of AmiC as a negative regulator of amidase expression is investigated using the *amiC* expression vector pSW41 and the purified protein.

CHAPTER FIVE - COMPLEMENTATION STUDIES IN *E.coli* AND *P.aeruginosa* AND INVESTIGATION OF AmiC FUNCTION

5.1 Introduction

Complementation analysis has been used extensively in previous studies of the amidase system (Drew, 1984; Cousens *et al*, 1987). This type of analysis has allowed the characterisation of a large number of amidase mutant strains in terms of their *amiE* and *amiR* genotypes. Complementation analysis also confirmed that *amiR* positively controlled amidase expression (Drew, 1984). In Chapter three the *amiC* gene was identified, and it was suggested this gene negatively regulated amidase expression. To test this hypothesis the *amiC* expression vector pSW41 was introduced into amidase constitutive strains of *P.aeruginosa* to investigate the role of AmiC as a negative regulator of amidase expression and allow further characterisation of the amidase constitutive strains in terms of their *amiC* genotype.

In addition the *amiR* expression vector pSW40 was introduced into *P. aeruginosa* strains to further investigate the role of *amiR* in amidase regulation. Finally, the purification of AmiC enabled experiments to be carried out *in vitro*, to investigate the mode of action of this regulatory protein.

5.2 Complementation analysis with pSW40, 41 and pSW42 in *P.aeruginosa*

Since AmiR could not be detected by the SDS-PAGE analysis described in Chapter four it was decided to investigate to what extent this vector produced AmiR by transcomplementation analysis. Several other *amiR* plasmids had been used in complementation analysis previously and it was hoped this type of experiment would provide a useful comparison.

In Chapter three two plasmids were described, pSW36 and pSW37 which showed that the newly identified gene *amiC* acted as a negative controlling element since mutations in the gene rendered the wild type inducible genes constitutive. Until the discovery of *amiC* it had been assumed that amidase constitutive mutants had mutations in the *amiR* gene. To test whether these previously characterised constitutive mutants were *amiC*$^-$, the broad host range

amiC expression vector (pSW41), was mobilised into a representative selection of constitutive mutant strains.

The SDS-PAGE analysis of pSW42 in strain PAC452 described in Chapter four was an attempt to identify any gene product encoded by the DNA sequence between *amiE* and *amiC*. This analysis failed to provide such evidence, mainly because the detection method was very insensitive. Since it had been shown that phage D3112 insertions in this region of DNA rendered PAO1 amidase negative (Rehmat and Shapiro, 1983), it was decided to introduce pSW42 into PAC1 and measure the affect on amidase expression. The rationale for this was that a transcomplementation system should be much more sensitive than looking for a novel band on a protein gel. Any alterations in amidase expression from PAC1 in the presence of pSW42 would indicate this region of DNA encoded a gene involved in amidase regulation.

5.2.1 Complementation with pSW40 in PAC327 and PAC1

PAC327 is an *amiR*$^-$ mutant derived from strain PAC142 (RED, unpublished). Strain PAC142 produces amidase constitutively at high levels (average = 28 units) and has an unlinked mutation to catabolite repression resistance (Potts and Clarke, 1976). PAC327 has been used previously in complementation analysis with plasmid pDC35, the homologue of pSW35 but carrying the 1.5kb *Xho*I *amiR* gene fragment from PAC433 (Cousens, 1985). The PAC327 pDC35 transcomplementation system showed constitutive amidase expression with an average specific activity of 12.25 units. Since amidase expression can be restored by transcomplementation with pDC35 this shows that PAC327 is *amiR*$^-$ and since it was derived from a constitutive mutant it is likely that it is also *amiC*$^-$. The average amidase activity seen with PAC327 pDC35 is only 42% of that seen with the parental strain PAC142. This could be due to poor transcription of *amiR* from the vector neomycin phosphotransferase II promoter. Alternatively, the fact that AmiR is not necessarily being produced close to its site of action could be reducing the level of transcomplementation, particularly if it has a short half life.

Plasmid pSW40 was mobilised into strains PAC327 and the wild type strain PAC1 by a triparental mating with the helper plasmid pRK2013 and amidase expression was measured under non-inducing, inducing and repressing conditions in the presence and absence of IPTG. The results of this transcomplementation analysis are shown in Table 5.1.

##########

TABLE 5.1. Amidase activities[a] from *P.aeruginosa* strains carrying the *amiR* expression vector pSW40

			Growth Conditions				
Strain	Plasmid	IPTG	Succinate	Succinate/ Lactamide	Lactate	Lactate/ Lactamide	Lactate/ Butyramide
PAC1	-	-	0.1	8.1	0.1	2.5	0.0
PAC1	pSW40	-	0.8	4.6	0.7	4.6	0.3
PAC1	pSW40	+	8.1	6.6	9.0	8.2	4.2
PAC142	-	-	30.8	23.3	16.2	31.3	22.0
PAC327	-	-	0.42	0.32	0.20	0.26	0.50
PAC327	pSW40	-	5.3	3.7	2.6	2.6	3.3
PAC327	pSW40	+	23.8	17	26.3	27.6	16

a) Amidase activity was measured in intact cells using the transferase assay (Brammar & Clarke, 1964). Cells were incubated overnight at 37°C unless otherwise indicated. Amidase activity is expressed as μmoles acethydroxamate formed per minute per mg bacteria. Values expressed are the mean of duplicate assays from at least three separate experiments.

##########

The wild type strain PAC1 shows inducible amidase activity with an average induced activity of 5.3 units. When plasmid pSW40 is mobilised into

strain PAC1 and grown in the absence of IPTG then an inducible phenotype is seen, but there is a low level of amidase expression even under non-inducing conditions. The average activity under non-inducing conditions is 0.6 units which is 11.3% of the average induced level for PAC1. This background level of expression is similar in percentage terms to the background expression seen with PAC327 pSW40 (see below) and indicates incomplete repression of the *tac* promoter. In the presence of IPTG, PAC1 pSW40 shows constitutive amidase activity with an average value of 7 units. Since the chromosomal *amiR* gene present in PAC1 can produce an average induced amidase activity of 5.3 units, the contribution from pSW40 in this transcomplementation system is small when amide inducer is present. This result provides evidence for AmiR being produced at saturating levels from pSW40. In the absence of amide inducer and presence of IPTG, PAC1 pSW40 produces 160% of the activity seen with PAC1 induced, which confirms the result with pDC5 pSW35 which showed that amide inducer was not required for AmiR to function as a transcription anti-terminator.

Strain PAC142 shows high constitutive levels of amidase activity, with an average specific activity of 28 units and the *amiR*$^-$ derivative PAC327 shows low constitutive levels of activity, with an average specific activity of 0.3 units. With strain PAC327 pSW40 grown in the absence of IPTG, low constitutive amidase expression is see under all growth conditions (average = 3.5 units). This level of expression represents 13% of the activity seen with the parental *amiR*$^+$ strain PAC142. This could be due to incomplete repression of the *tac* promoter in pSW40 as found with PAC1 pSW40 (see above).

When PAC327 pSW40 is grown in the presence of IPTG there is a six fold increase in the amount of amidase produced under all growth conditions (average = 22.4 units). This level of expression is comparable with the activity seen from the parental strain PAC142 (average 28 units). However, the increase in activity is small compared with values reported by other workers (Bagdasarian *et al*, 1983) who report up to a twenty fold increase in expression from

the *tac* promoter in the presence of IPTG. This suggests that AmiR might be present at saturating concentrations, which is feasible as positively acting regulatory proteins are often only required in small quantities (Raibaud and Schwartz, 1984). A similar result was found in PAC1 with pSW40 grown in the presence of IPTG (see above)

5.2.2 Complementation with pSW41 in amidase constitutive *Pseudomonas* strains

Until the discovery of *amiC* two possible reasons had been considered for *P.aeruginosa* producing amidase constitutively. Firstly, mutations in the *amiR* gene or secondly mutations in the transcription terminator upstream of *amiE* rendering it inactive. The identification of *amiC* immediately meant the genotype of the large number of constitutive mutants that had been isolated had to be reassessed, since it was likely that a large number of these mutants were *amiC* $^-$. This was now possible using the *amiC* expression vector pSW41 and so this plasmid was mobilised into three amidase constitutive *P.aeruginosa* strains : - PAC101, PAC111 and PAC433. The family history of these three strains is shown below:-

PAC1 (wild type, inducible) —spontaneous→ PAC111 (constitutive, butyramide sensitive)
↓
EMS
↓
PAC101 (constitutive, butyramide resistant)
↓
NMG
↓
PAC432 (amidase negative, *amiR*$^-$)
↓
NMG
↓
PAC433 (constitutive, catabolite repression resistant)

Amidase activity was assayed in the three constitutive mutants containing pSW41 under inducing, non-inducing and repressing conditions in the presence and absence of IPTG and the results are shown in Table 5.2 along with amidase activities from the three strains alone.

PAC101 is the original constitutive parent of the magnoconstitutive

TABLE 5.2. Amidase activities[a] from amidase constitutive *P.aeruginosa* strains carrying the *amiC* expression vector, pSW41

Strain	Plasmid	IPTG	Growth Conditions: Succinate	Succinate/ Lactamide	Lactate	Lactate/ Lactamide	Lactate/ Butyramide	Assay
PAC101	-	-	17.8	11.3	8.8	3.5	7.3	
PAC101	pSW41	-	3.5	2.0	1.6	1.8	3.4	
PAC101	pSW41	+	2.8	2.1	2.9	1.5	1.2	
PAC111	-	-	6.7	9.2	2.7	3.4	0.1	
PAC111	pSW41	-	0.5	0.4	0.1	1.2	0.1	
PAC111	pSW41	+	0.4	0.2	0.3	0.6	0.1	
PAC433	-	-	57.2	51.1	30.8	33.4	26.2	
PAC433	pSW41	-	35.0	6.4	2.28	4.54	4.45	1.
PAC433	pSW41	-	33.0	21.0	20.0	12.0	24.0	2.
PAC433	pSW41	-	1.3	7.11	1.6	1.9	0.6	3.
PAC433	pSW41	-	1.2	1.2	9.66	1.04	14.2	4.
PAC433	pSW41	+	4.7	1.4	15.0	1.3	17.3	1.
PAC433	pSW41	+	88.5	7.0	29.0	29.0	23.0	2.
PAC433	pSW41	+	3.0	3.5	0.8	2.2	0.7	3.
PAC433	pSW41	+	4.4	16.3	10.2	7.6	1.5	4.

a) Amidase activity was measured in intact cells using the transferase assay (Brammar & Clarke, 1964). Amidase activity is expressed as μmoles acethydroxamate formed per minute per mg bacteria. The values shown above the broken line represent the mean of duplicate assays carried out on at least three separate occasions. The values below the broken line represent the values of individual assays, with each line representing values obtained on separate days.

strain PAC433, from which the amidase genes have been cloned (Brammar *et al*, 1967). It produces high levels of amidase under all growth conditions with an average specific activity of 9.7 units. In the presence of pSW41, the activity from PAC101 is reduced by 75% in the absence of IPTG and 79% in the presence of IPTG. Thus AmiC is repressing amidase expression from this constitutive host. The repression by AmiC occurs even in the absence of IPTG, but the crude extract analysis in Chapter four of PAC452 pSW41 has previously shown that this plasmid produces large amounts of AmiC without IPTG induction. Interestingly, AmiC repression by pSW41 in PAC101 is not relieved by amide inducer

PAC111 is a spontaneous constitutive mutant derived from PAC1 which remains sensitive to butyramide repression. The average amidase activity produced from this strain is 5.5 units (excluding butyramide activity). In the presence of pSW41 there is a ten fold reduction in amidase activity (7% residual activity) which is independent of IPTG induction. As with PAC101 pSW41, AmiC repression of amidase expression is not relieved by lactamide.

PAC433 was the strain chosen to originally clone the amidase genes (Drew *et al*, 1980), since it produces high levels of amidase under all growth conditions (average activity = 40 units). This strain has a linked mutation to catabolite repression resistance which probably contributes to the high amidase activity produced. When pSW41 was introduced into this strain inconsistent amidase activities were seen. The results of the individual assays are shown in Table 5.2. Considering first PAC433 pSW41 grown in the absence of IPTG. With the first set of results there appears to be a ten fold decrease in amidase expression under all growth conditions except succinate. The second set of assays were performed four days later and only show a two fold reduction in amidase expression. These assays used single colonies picked from the same plate as for the first set of assays. After these two results, plasmid pSW41 was remobilised into PAC433 and assayed again very shortly after the mobilisations had been completed. The third and fourth sets of assays show the

results after this second mobilisation. In these assays there is up to a forty fold reduction in amidase activity however even with some of these assays some growth conditions only show a two fold reduction.

The results with PAC433 pSW41 grown in the presence of IPTG really parallel the results obtained in the absence of IPTG. It would appear that when pSW41 is mobilised into PAC433, functional AmiC production is unstable, since new pSW41 transconjugants show almost complete repression of amidase activity, but after storing the strains for 3-4 days, certain cultures start to show derepression and produce amidase. Since the derepression of amidase expression appears to occur randomly within any set of assays, this suggests that spontaneous mutations are occurring in *amiC* in pSW41 which have a selective growth advantage.

Plasmid DNA was isolated from some cultures which had started expressing amidase at high levels and restriction mapped. There was no evidence of deletions accumulating in these plasmids, therefore any mutations which prevent pSW41 from producing functional AmiC must have been either very small deletions or point mutations. It is known that AmiC production from pSW41 is constitutive and at very high levels and that mutations in this plasmid appear to accumulate quickly, preventing functional *amiC* expression. This would imply that over production of AmiC is deleterious to the cell and there is a positive selection against it. This was also found to be the case with pSW26 in *E.coli* (Chapter 4).

Further assays done on PAC101 and PAC111 containing pSW41 when the cells had been stored on a plate for two weeks also showed relief of AmiC repression in some assays. From these complementation assays it can be seen that amidase expression from all three amidase constitutive mutants can be repressed by *amiC*, implying that these mutants were indeed *amiC* $^-$ and *amiC* is involved in the regulation of induction of amidase. One mystery arose from these transcomplementations and that was why lactamide could not relieve repression by pSW41. Assuming inducer normally interacts with AmiC, preventing

repression of amidase expression, then it could be that insufficient inducer is getting into the cells to bind and inactivate the large amount of AmiC produced by pSW41. Previous studies have indicated there is no active transport system for amides in *P.aeruginosa* (Farin, 1976), therefore the diffusion of lactamide into the cells might be unable to match AmiC production consequently amidase expression within the cells would be permanently repressed.

5.2.3 Complementation with pSW42 in PAC1

Plasmid pSW42 was mobilised from *E.coli* JA221 into the wild type *Pseudomonas* strain PAC1 by a triparental mating with the helper plasmid pRK2013. Transconjugants were isolated and amidase assays were performed under non-inducing, inducing and repressing conditions, in the presence and absence of IPTG. The results are shown in Table 5.3. With PAC1 pSW42 with and without IPTG an inducible phenotype is seen with an average induced activity of 2.5 units This level of expression is two fold lower than the average induced level seen with PAC1 (5.3 units). Whether the reduction in amidase activity is significant is difficult to assess.

****

TABLE 5.3 Amidase activity[a] from *P.aeruginosa* strain PAC1 carrying plasmid pSW42

			Growth Conditions				
Strain	Plasmid	IPTG	Succinate	Succinate/ Lactamide	Lactate	Lactate/ Lactamide	Lactate/ Butyramide
PAC1	-	-	0.1	8.1	0.1	2.5	0.0
PAC1	pSW42	-	0.0	1.8	0.0	4.2	0.0
PAC1	pSW42	+	0.1	2.3	0.2	2.7	0.0

a) Amidase activity was measured as μmoles acetohydroxamate produced per minute per mg bacteria. The values presented are the means of duplicate assays carried out on at least three separate occasions.

****

PAC1 has an identical chromosomal copy of the DNA present on pSW42, therefore the provision of extra copies of this DNA sequence might not be expected to alter amidase expression significantly. The results from this transcomplementation are inconclusive. The phage D3112 insertions in this region of DNA rendered the system amidase negative, suggesting this region of DNA might encode a positive regulator (Rehmat and Shapiro, 1983), yet when excess copies of this region of DNA are provided there is a decrease, not an increase, in amidase activity. Further analysis of this region of DNA is described in Chapter six.

5.2.4 Construction of pSW101 and its amidase activity in strain PAC452

Amidase expression from pAS20 in *E.coli* is virtually undetectable even under inducing conditions and thus it has not been shown that the 5.3kb *Hin*dIII-*Sal*I fragment cloned from PAC1 actually contains all the genes required for normal inducible amidase expression. Since the amidase levels in *E.coli* are so low it is very difficult to devise an experiment to show that all the genes involved in amidase induction have been cloned. Additionally, by introducing the individually subcloned genes into a *P.aeruginosa* host it could be argued that transcomplementation is occurring with unlinked genes involved in the induction process on the chromosome. Despite these problems it was decided to introduce the wild type cloned genes into an amidase deletion *P.aeruginosa* host and investigate amidase expression. The *Hin*dIII-*Sal*I fragment from pAS20 was subcloned into the broad host range plasmid pKT231. The resulting plasmid, pSW101, was characterised by restriction mapping with *Hin*dIII, *Sal*I, *Xho*I and *Pvu*II. A restriction map of this plasmid is shown in Figure 5.1.

pSW101 was mobilised into the Ami deletion strain PAC452 by triparental matings with the helper plasmid pRK2013. Amidase activity was measured under inducing, non-inducing and repressing conditions and the results are shown in Table 5.4. An inducible phenotype is seen with an average induced activity of 40 units. This level of expression is eight times higher than that

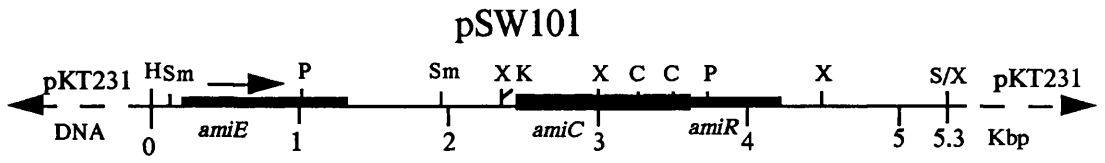

Figure 5.1. Partial restriction map of plasmid pSW101. This plasmid was generated by subcloning the *Hin*dIII-*Sal* I fragment from pAS20 into the *Hin*dIII and *Xho* I targets of the broad host range cloning vector pKT231. Abbreviations for restriction enzyme targets are as follows: H= *Hin*dIII, P= *Pvu*II, X= *Xho*I, K= *Kpn*I, C= *Cla*I, S= *Sal* I, Sm = *Sma* I, S/X refers to the hybrid site generated by ligation of *Sal* I and *Xho* I cohesive ends.

Table 5.4. Amidase activities from plasmid pSW101 in the Ami deletion *P.aeruginosa* strain PAC452.

Strain	Growth Conditions				
	Succinate	Succinate Lactamide	Lactate	Lactate Lactamide	Lactate Butyramide
PAC452 pSW101	1.1	51.9	0.6	30.7	0.9

Amidase activity was measured as micromoles acetohydroxamate produced per minute per mg bacteria. Values presented are the mean of duplicate assays carried out on at least three separate occasions.

seen with PAC1 under similar inducing conditions. This result is presumably because of the multiple copies of the amidase genes are present in the cell (copy number of pKT231 = 13, Frey and Bagdasarian, 1989). A background level of amidase expression is seen under non-inducing conditions, which is also probably because of the copy number of pSW101.

This experiment does not prove that the 5.3kb *Hin*dIII-*Sal*I fragment contains all the genes required for normal amidase regulation (excluding global controls) because there could be a chromosomally unlinked gene involved, provided *in trans* by PAC452. However, an unlinked gene involved in amidase regulation would only be present as a single copy, whereas there are approximately 13 copies of the amidase genes. This imbalance might disturb the induction process, yet a normal inducible phenotype is seen. This would argue in favour of the 5.3kb *Hin*dIII-*Sal*I fragment containing all the genes required for inducible amidase expression. Further experiments described in Chapter six confirm this hypothesis.

5.3 DNA binding assays with purified AmiC

AmiC has been identified as a negative regulator of amidase expression by two lines of evidence: firstly, deletions within the gene cause constitutive amidase expression and secondly provision of AmiC *in trans* represses amidase expression from amidase constitutive mutants. Neither of these experiments gave any information on how AmiC represses amidase expression, nor did the experiments with the promoter probe vector, pSW10.

In Chapter three a model for AmiC repression of amidase synthesis was proposed, in which AmiC was a conventional DNA binding repressor. By this model, AmiC was expected to bind to an operator sequence adjacent to the *amiCR* promoter and regulate expression of itself and AmiR. This repression would be relieved by an inducer binding to AmiC and preventing it from binding to the operator sequence.

One expectation of this model would be that *amiCR* expression would

be regulated in response to inducer. To confirm this a preliminary dot blot was carried out with non-induced and induced RNA (20μg) isolated from PAC452 pSW101. The dot blot was probed with the 1.5kb *Xho*I fragment from pAS20 (^{32}P-labelled) which encodes the C-terminal region of *amiC*, and *amiR*. The results from this dot blot analysis are shown in Figure 5.2 This preliminary experiment indicated that *amiCR* transcription was increased in response to inducer, which was consistent with the model proposed, with AmiC acting as a conventional DNA binding repressor. Consequently, experiments were carried out using purified AmiC to determine whether it had DNA binding activity and these are described below.

5.3.1 Gel retardation assays with AmiC

Gel retardation assays are used to detect the binding of proteins to specific regions of DNA. The basis of the assay is that with small DNA molecules, their mobility in non-denaturing polyacrylamide gels is reduced when a protein is bound. To prevent the non-specific binding of proteins to a given sequence, a large excess (up to 1000 fold) of competitor DNA, such as Salmon sperm DNA, is added. The use of competitor DNA is not strictly necessary when a purified protein is used, if the protein is free from contaminants. Gel retardation assays (bandshifts) are only effective with relatively small DNA fragments (up to 1 kbp), since with large DNA fragments, which have low mobility in polyacrylamide gels, the difference in mobilities between protein bound and not bound are too small to detect.

If AmiC regulated transcription of *amiC* and *amiR*, it was assumed it would bind to an operator site close to the promoter elements identified within the 658bp *Xho*I fragment. Since one of these potential promoter elements lies very close to the 5' end of the 658bp *Xho*I fragment, it was decided to use a probe which started within the *amiC* coding region and extended upstream by 700bp, thus encompassing the two promoter elements and a considerable amount of flanking sequence. The fragment used was the 700bp *Sma*I (1900) to *Eco*RV (2600) fragment shown in Figure 5.3. To facilitate the

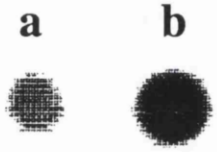

Figure 5.2. Dot-Blot analysis using RNA isolated from PAC452 pSW101 under non-inducing (a) and inducing (b) conditions. The Dot-Blot was probed with the 1.5kb *Xho*1 fragment from pAS20. This analysis indicates that *amiCR* transcription is increased in response to inducer.

labelling of this fragment the probe was isolated from pSW14 (*Sma*I(1900-*Pvu*II(3800) fragment from pAS20 subcloned into the multiple cloning site of pSW10) on a *Hin*dIII- *Eco*RV fragment which was then end-labelled using T4 polynucleotide kinase after dephosphorylation. *Sma*I and *Eco*RV both generate blunt ends which label very inefficiently with T4 polynucleotide kinase, whereas *Hin*dIII produces a 5' overhang which labels efficiently. Thus the probe contains the *P.aeruginosa* DNA sequence from *Sma*I to *Eco*RV in addition to 37 bases from the multiple cloning site (MCS) of pSW14 (the MCS was obtained from M13mp18).

Conventionally gel retardation assays are carried out using polyacrylamide gels. Recently though, agarose gels have been used successfully (Dale *et al,* 1989). Agarose gels are considerably easier to cast so it was initially decided to use a 1% agarose gel cast with 0.5X TBE. Since AmiC had been purified to homogeneity this purified protein was used in the assays. The amount of probe used, and the conditions of the reaction were as described in materials and methods for polyacrylamide gels. A variety of concentrations of AmiC were used in the reactions, along with a 1000 fold excess of competitor DNA. The results of this analysis are shown in Figure 5.3.

Lane 1 contains the free probe with a 1000 fold excess of cold competitor DNA and provides a control for the mobility of the labelled fragment. Lanes 2,3 and 4 contain the probe + competitor DNA and 15, 30 and 45ng of purified AmiC respectively. There is no evidence of reduced mobility of the probe in these lanes. Lanes 5 and 6 contain probe + competitor + 45ng AmiC + 0.5 and 1% lactamide respectively. Again there is no evidence of reduced mobility of the probe. Lane 7 is in effect a negative control. It contains probe + 30ng AmiC + 100ng of cold pAS20, which it was hoped would compete out the AmiC binding to the probe, preventing a bandshift.

These results provided no evidence for AmiC binding to the probe. Since the probe was actually quite a large fragment of DNA, any reduced mobility due to AmiC binding might have gone undetected on this agarose gel, but

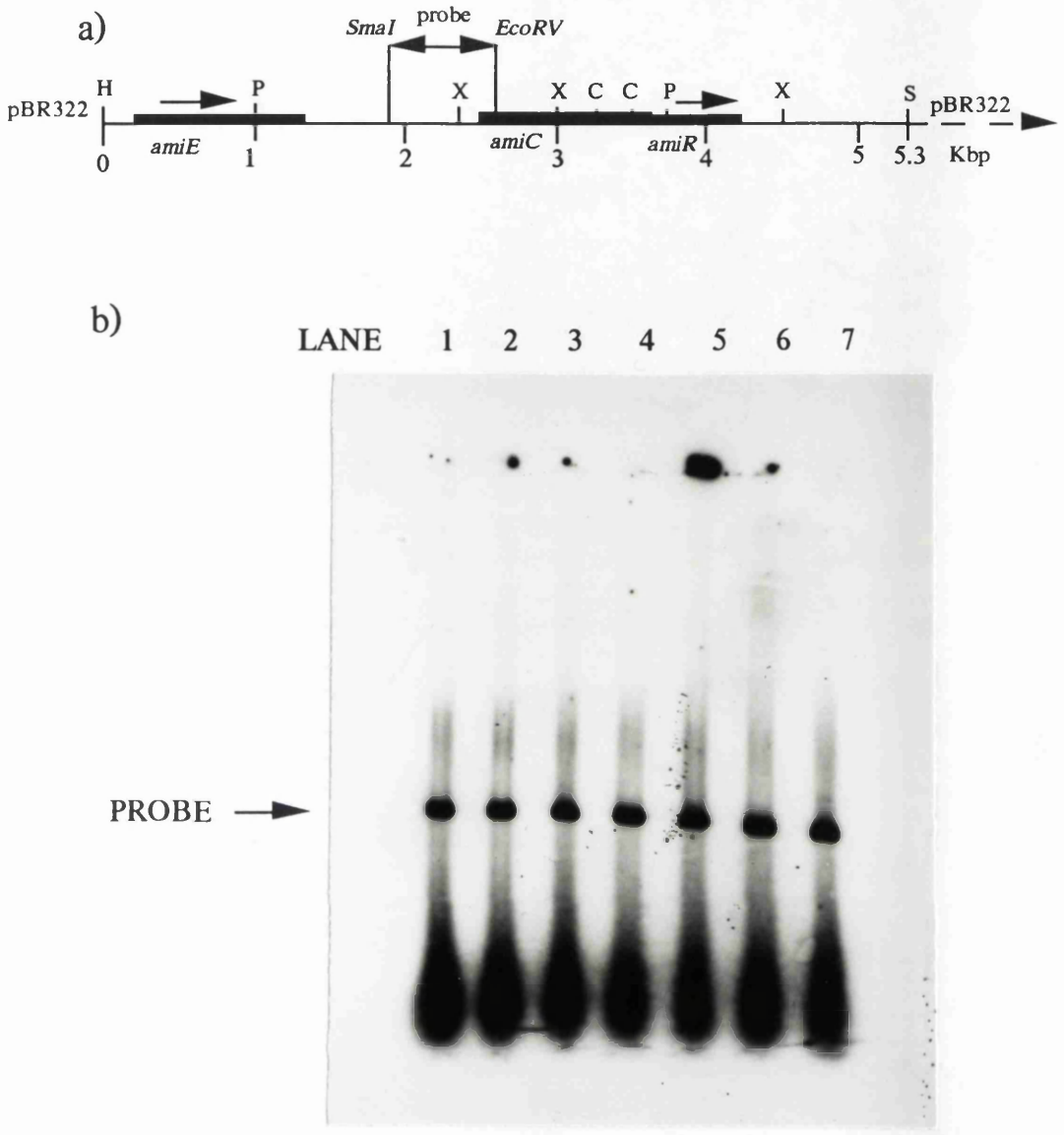

Figure 5.3. a) Location of the 700bp *Sma* I - *Eco*RV fragment used as a probe for the gel retardation assay shown above.

b) Gel retardation assay using purified AmiC and the probe described above. Samples were resolved on a 1% agarose gel .Lane 1, free probe; Lane 2, probe + 15ng AmiC; Lane 3, probe + 30ng AmiC; Lane 4 probe + 45ng AmiC; Lane 5, probe + 45ng AmiC + 0.5% lactamide; Lane 6, probe + 45ng AmiC + 1% lactamide; Lane 7, probe + 30ng AmiC + 100ng pAS20.

might show up on a polyacrylamide gel, which has a better resolving power for DNA fragments < 1 kb in size. The experiment was repeated using a 4% non-denaturing polyacrylamide gel, using the same probe.

For any given protein-DNA binding the experimental conditions for optimal binding of the protein to the DNA have to be determined. Generally the main variable to consider is the salt concentration at which the binding reaction is carried out (Perbal, 1988). In the second gel retardation assay, carried out with a polyacrylamide gel, the salt concentration in the binding reactions was varied and the results are shown in Figure 5.4. The binding reactions for the first four lanes were all carried in the presence of 40mM NaCl and 1000 fold excess of competitor DNA (Salmon sperm DNA).

The first lane contains the free probe with no protein added. A single band is seen, however there is a higher molecular weight smear, which is some form of contaminant. Lanes 2,3 and 4 contain the probe + competitor + 15, 30 and 45ng of purified AmiC respectively. The competitor was used to avoid non-specific probe binding by any contaminants that might be present in the purified AmiC extract. There is no evidence of reduced mobility of the probe in any of these lanes, suggesting AmiC is not binding to the probe. Lane 5 contains 45ng purified AmiC but no competitor. Some of the probe has not migrated into the gel. This indicates that contaminants in the AmiC extract are binding non-specifically to the probe and retarding its mobility to such an extent that it cannot enter the gel. Lane 6 contains probe + 1000 fold excess of competitor + 45ng AmiC and 100ng pAS20, added as a specific competitor for AmiC binding. No bandshift is seen. Lanes 7,8 and 9 contain probe + 1000 fold excess of competitor + 45ng AmiC and 60, 80 and 100mM NaCl respectively. There is no evidence of reduced mobility of the probe. The overwhelming conclusion drawn from these two experiments was that AmiC does not appear to bind to the *Sma*I- *Eco*RV fragment used as the probe. Two other gel retardation assays were carried out before abandoning the technique. In the

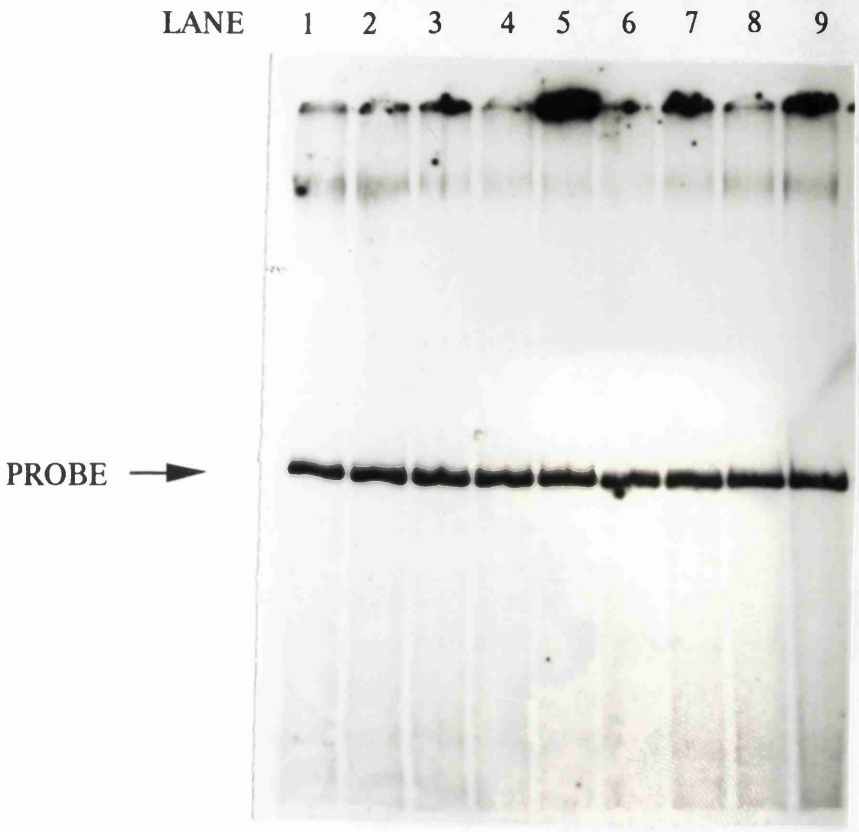

Figure 5.4. Gel retardation assays using the 700bp *Sma* I- *Eco*RV probe and purified AmiC. Samples were resolved using a 4% non-denaturing polyacrylamide gel. Lane 1, free probe; Lane 2, probe + 15ng AmiC (40mM NaCl); Lane 3, probe + 30ng AmiC (40mM NaCl); Lane 4 , probe + 45ng AmiC (40mM NaCl); Lane 5, probe + 45ng AmiC (no competitor DNA); Lane 6, probe + 45ng AmiC + 100ng pAS20; Lane 7, probe + 45ng AmiC (60mM NaCl); Lane 8, probe + 45ng AmiC (80mM NaCl); Lane 9, probe + 45ng AmiC (100mM NaCl).

first one, the amount of AmiC was kept constant and the amount of competitor was varied from a 10 fold excess to a 1000 fold excess. Once again there was no evidence of AmiC binding to the probe.

At the time these experiments were carried out a paper was published on gel retardation assays with the *E.coli trp* repressor (Carey, 1988). In this paper it was shown that the ability of *trp* repressor to retard the electrophoretic mobility of an operator DNA fragment depended on the pH of the gel system. Above the pI of the protein, little retardation of DNA was observed. As the pH of the gel was lowered, retardation was enhanced and could be detected. The reason for this effect was that at pH values above the pI of the protein, TrpR had a mobility in the gel almost equivalent to that of the DNA and hence provided little retardation effect.

Since the predicted pI of AmiC is 6.7 and the gel system that had been used was pH 8.0, it was possible that no retardation effect had been seen with AmiC bound to the probe for the reason given above. Consequently a gel/buffer system, identical to the one used for the *trp* repressor (Carey, 1988) was used for the final set of gel retardation experiments. Even with this system there was no evidence of reduced mobility of the probe in the presence of AmiC (data not presented).

There were two main problems associated with the gel retardation experiments described above; firstly, there was no positive control, because it would be very difficult to devise a meaningful one, and secondly the experiments relied upon the assumption that AmiC bound to the small fragment of DNA used as the probe. Consequently a second technique (filter binding assays) was employed, to try and detect DNA binding by AmiC.

5.3.2 Filter binding assays

Filter binding assays rely on the fact that double stranded nucleic acids do not bind to nitrocellulose filters. However proteins will bind and

consequently if a protein binds to fragment of DNA this will cause the DNA to be retained on the filter (Riggs *et al*, 1970). Therefore by measuring the retention of a radioactively labelled piece of DNA on a filter, this indicates whether protein is binding to the DNA. Competitor DNA is added to the binding reactions to prevent non-specific binding of proteins to the test DNA fragment.

The advantage with filter binding assays over gel retardation assays is that there is no size limit to the test DNA fragment used, consequently it was possible to carry out filter binding assays with the whole 5.3kb *Hin*dIII-*Sal*I fragment of *P.aeruginosa* DNA containing the amidase genes.

Filter binding assays were carried out with pAS20 which had been linearised at the unique *Hin*dIII target and end-labelled, as described in Chapter two. The results of one such experiment is presented in Table 5.5.

##########

TABLE 5.5 - Filter binding assays with pAS20 DNA and purified AmiC.

sample	% retention on filter*
^{32}P-pAS20 + competitor	16.8 (background)
^{32}P-pAS20 + competitor + .75µg AmiC	20.7
^{32}P-pAS20 + competitor + 1.5µg AmiC	15.1
^{32}P-pAS20 + competitor + 3µg AmiC	11.4
^{32}P-pAS20 + competitor + 4.5µg AmiC	14.8
^{32}P-pAS20 + competitor + 1% lactamide + 1.5µg AmiC	15.1
^{32}P-pAS20 + 1µg cold pAS20 + 1.5µg AmiC	13.9

* - each sample contained the same amount of ^{32}P labelled pAS20, and the retention figures represents how much of the radiolabelled pAS20 was retained on the filters as determined by liquid scintillation counting.

##########

The results indicate that the presence of up to 5μg of purified AmiC does not significantly increase the retention of the radioactively labelled pAS20 on the nitrocellulose filters, indicating AmiC does not have any DNA binding activity. Several other filter binding experiments were carried out varying the amount of competitor DNA, amount of AmiC, and even using crude extracts containing AmiC and none of these experiments showed any evidence for AmiC having DNA binding activity. A final experiment was carried out to confirm that AmiC was not a conventional repressor acting at the *amiE* promoter and this is described below.

5.3.3 Construction of pSW41G and transcomplementation with pCL55

A transcomplementation system was established in *E.coli* using plasmids pCL55 and pSW41G. pCL55 (Drew and Lowe, 1989) is a derivative of plasmid pJB950 (PAC433 genes) missing all *Pseudomonas* DNA sequences downstream of the first *Xho*I target (2400) and deleted for the 10bp *Sma*I fragment in the transcription terminator upstream of *amiE* (Figure 5.4). pCL55 expresses amidase at high constitutive levels, independently of AmiR and carries the ampicillin resistance gene of pBR322. To investigate the effect of AmiC on amidase expression from pCL55, a gentamycin resistance gene (2.3kb *Eco*RI fragment from plasmid pQR139) was cloned into the ampicillin resistant *amiC* expression vector pSW41. The resultant plasmid, pSW41G (Figure 5.5), now Amp^R Gm^R was transformed into *E.coli* JA221 carrying pCL55 and the resultant strain was assayed for amidase activity under inducing, non-inducing and repressing conditions. The results of these assays are shown in Table 5.6

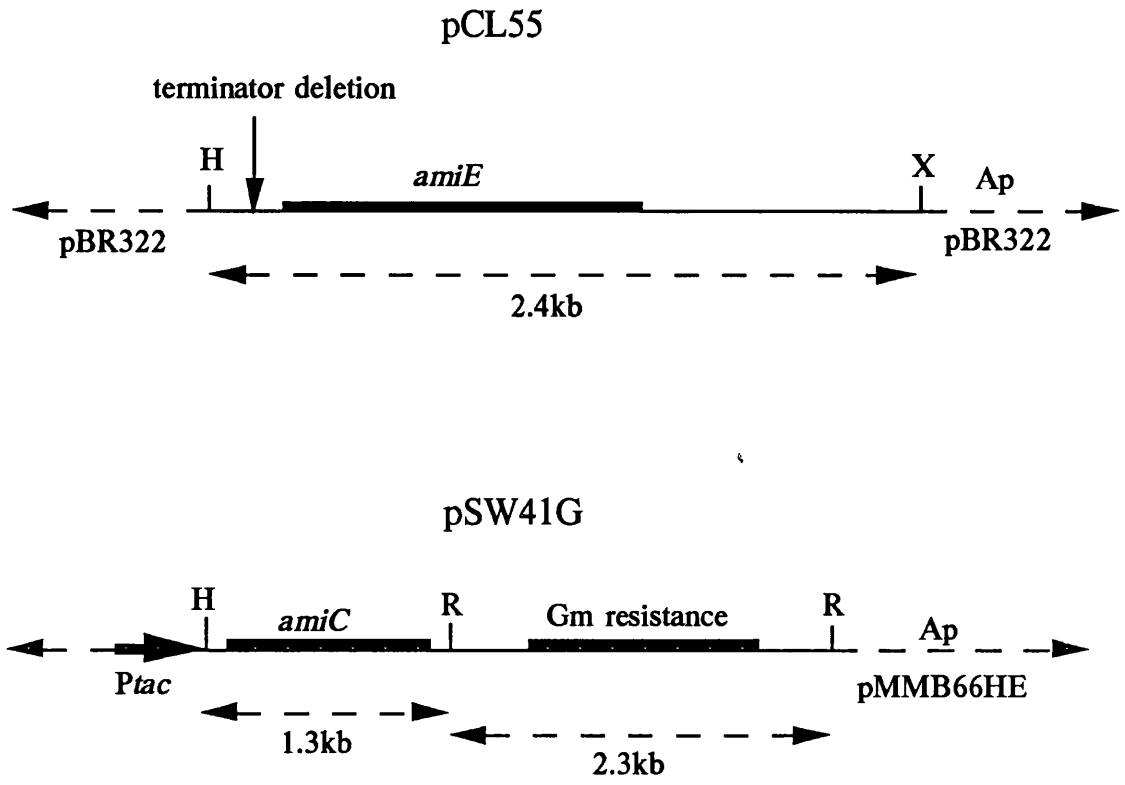

Figure 5.5. Restriction maps of plasmids pCL55 (Drew and Lowe, 1989) and pSW41G. The maps are not drawn to scale. pCL55 has a 10bp deletion in the terminator upstream of *amiE* and expresses *amiE* constitutively. pSW41G is a derivative of pSW41 and contains *amiC* subcloned downstream of the *tac* promoter in the broad host range expression vector pMMB66HE. In addition a gene encoding resistance to gentamycin has been subcloned into the unique *Eco* RI target downstream of *amiC* to generate plasmid pSW41G. Restriction target abbreviations are as follows:- H = *Hind* III, R= *Eco* RI and X = *Xho* I.

TABLE 5.6 Amidase activities[a] from *E.coli* strain JA221 containing plasmids pCL55 and pSW41G

Plasmids	IPTG	Growth Conditions				
		Glucose	Glucose/ Lactamide	Succinate	Succinate/ Lactamide	Succinate/ Butyramide
pCL55	-	20.8	35.2	40.4	37.0	42.6
pCL55/pSW41G	-	51.2	48.8	54.4	54.6	62.8
pCL55/pSW41G	+	42.0	42.8	53.1	54.2	56.0

a) Amidase activity was measured as μmoles acetohydroxamate produced per minute per mg bacteria. Values presented are the mean values of duplicate assays carried out on at least three separate occasions.

Plasmid pCL55 shows high constitutive amidase expression with an average specific activity of 35.2 units. In the presence of pSW41, in both the presence and absence of IPTG, there is no decrease in amidase expression from pCL55, in fact there is a slight increase. This result confirms that AmiC does not repress amidase expression by interacting with the *amiE* promoter.

5.4 Evidence that AmiC represses AmiR by a protein-protein interaction

The gel retardation assays and the filter binding studies failed to show any evidence for AmiC having DNA binding activity. Thus the first hypothesis that AmiC was a conventional DNA binding repressor was rejected.

A second model was proposed in which AmiC might repress the anti-termination activity of AmiR by a protein-protein interaction. This repression could occur by two alternative ways. AmiC could bind to AmiR directly to prevent its antitermination activity. Alternatively, AmiC repression of AmiR might be mediated by a covalent modification of AmiR, such as phosphorylation.

To investigate this second model in which AmiC repression of AmiR occurred at the protein level a three plasmid transcomplementation system was set up in *E.coli*. The experimental design for this transcomplementation system was as follows: Firstly an *amiE* containing plasmid was constructed (pTM1) which carried the *amiE* gene, its normal promoter and transcription terminator. This plasmid was made to provide an assay for functional AmiR. Secondly, a compatible plasmid was constructed containing *amiR* transcribed constitutively from the *lac* promoter (pTM2). Transcription of *amiR* from this plasmid should not be effected by *amiC*. Thirdly plasmid pSW41 which produces AmiC would be introduced into *E.coli* cells containing the other two plasmids. If the presence of pSW41 caused a reduction in the transcomplementation levels between the *amiE* and *amiR* plasmids this would indicate that AmiC repressed the antitermination activity of AmiR by some form of protein-protein interaction. The construction of plasmids pTM1 and pTM2 and the complementation assays with these plasmids were carried out under my supervision by a final year project student, Tahir Malik. I would like to thank him for his technical contribution to these experiments.

5.4.1 Construction of pTM1

For the three plasmid transcomplementation to work, it requires that the three plasmids have different and compatible origins of replication and also have different selectable markers. To construct the *amiE* containing plasmid, a derivative of plasmid pAS25 was made. pAS25 contains the *Hin*dIII-*Sal*I fragment from pJB950 subcloned into the cloning vector pACYC184. Consequently pAS25 contains a P15A replicon (Chang and Cohen, 1978) and chloramphenicol resistance as a selectable marker. This plasmid was digested with *Xho*I to remove *amiC* and *amiR* sequences, and was religated and used to transform *E.coli* JA221 to chloramphenicol resistance. A restriction map of the parental plasmid pAS25 and the newly constructed *amiE* containing derivative, pTM1, is shown in Figure 5.6.

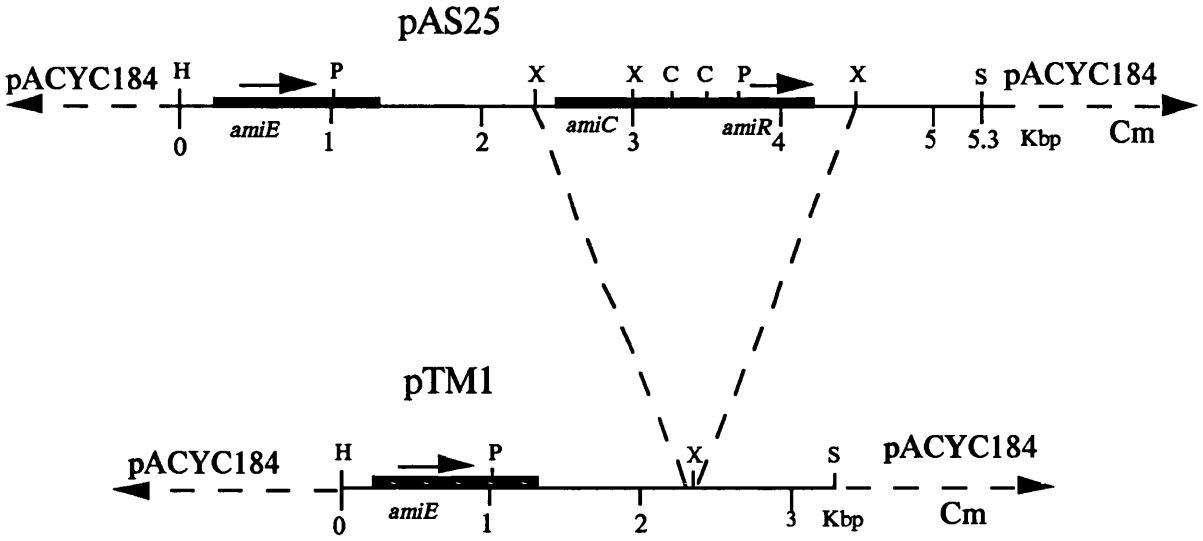

Figure 5.6. Restriction maps of plasmids pAS25 and its derivative pTM1. Abbreviations are as follows: H= *Hind* III, P= *Pvu* II, X= *Xho* I, C= *Cla* I, S= *Sal* , Cm = chloramphenicol resistance.

A key feature of this experiment was that transcomplementation between the *amiR* containing plasmid and pTM1 (*amiE*) would be easily detectable by an amidase assay. To test this pTM1 was transformed into *E.coli* strains containing two previously constructed plasmids which expressed *amiR*.

5.4.2 Complementation analysis with pTM1, pSW24 and pSW40

The two *amiR* plasmids to be used in the transcomplementation with pTM1 were pSW24 and pSW40 (see Chapter 4). *amiR* should be transcribed constitutively from the *lac* promoter in pSW24 and its' expression is regulated by IPTG in pSW40. The results of the transcomplementation assays are shown in Table 5.7 together with assays of pTM1 and pAS25 alone.

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TABLE 5.7. Amidase activities[a] from *E.coli* strain JA221 containing plasmids pAS25, pTM1, pSW40 and pSW24.

		Growth Conditions				
Plasmids	IPTG	Glucose	Glucose/ Lactamide	Succinate	Succinate/ Lactamide	Succinate/ Butyramide
pAS25	-	0.96	0.97	0.79	0.86	0.82
pTM1	-	0.51	0.46	0.44	0.70	1.29
pTM1/pSW24	-	35.5	22.4	27.3	28.2	19.8
pTM1/pSW24	+	27.4	26.1	19.1	15.1	25.3
pTM1/pSW40	-	0.83	0.62	0.86	0.68	0.83
pTM1/pSW40	+	3.5	3.5	12.6	11.6	11.6

a) Amidase activity was measured as μmoles acetohydroxamate produced per minute per mg bacteria. The values presented are the mean of duplicate assays carried out on at least three separate occasions.

****

pAS25 shows low constitutive amidase activity under all growth conditions. The level of activity is approximately 25% of that seen with pJB950, and probably reflects the lower copy number of pAS25 (10-20) compared to pJB950 (40-50, H. Richards, personal communication). pTM1 shows a background level of amidase expression with an average specific activity of 0.68 units. In the presence of pSW24, high levels of amidase activity are seen from pTM1 (average = 26.6 units) and this level is not increased by the addition of IPTG (average with IPTG = 22.6 units). This confirms that *amiR* expression from pSW24 is constitutive and AmiR functions independently of inducer. When pSW40 is introduced into JA221 containing pTM1 and grown in the absence of IPTG a low constitutive level of amidase is seen, similar to the level seen with pTM1 alone, suggesting complete repression of the *tac* promoter in pSW40 in *E.coli*. In the presence of IPTG, amidase is produced constitutively, with an average specific activity of 8.7 units. This level of expression is 33% of the level seen with pSW24 pTM1 and is probably due to the lower copy of pSW40 compared to pSW24.

Interestingly the levels of activity seen when pTM1 pSW40 + IPTG are grown in the presence of glucose are approximately 25% of the levels seen in succinate media. This suggests a catabolite repression effect is occurring, since glucose is the preferred carbon source for *E.coli* whereas succinate is a poor carbon source. It is most probable that this effect is occurring in pSW40 since *amiE* expression from recombinant plasmids has previously been shown not be catabolite repressed in *E.coli* (R. Drew, personal communication). These complementation experiments have shown that high levels of amidase can be produced when *amiR* is expressed from the *lac* promoter in pSW24 which has a ColE1 origin of replication and thus is compatible with pTM1 and pSW41 (RSF1010 origin of replication). However, the selectable marker on pSW24 is ampicillin resistance, which is also the marker for pSW41. Therefore a different *amiR* plasmid had to be constructed, with the same origin of replication as pSW24 but a different selectable marker. This is described below.

5.4.3 Construction of pTM2

pSW24 is a derivative of pUC19 and has the *bla* gene conferring ampicillin resistance on the host. Recently an analoguous series of plasmids to the pUC series have been constructed in which the *bla* gene has been substituted for a gene conferring resistance to kanamycin on the host (pBGS series, Spratt *et al,* 1986). Therefore the 1.5kb *Hin*dIII-*Eco*RI fragment from pSW24 was subcloned into the *Hin*dIII and *Eco*RI sites pBGS19$^-$ to generate plasmid pTM2. A restriction map of this plasmid is shown in Figure 5.7. The orientation of the *amiR* gene with respect to the *lac* promoter is the same as for pSW24. This plasmid was used in the final set of experiments in conjunction with pTM1 and pSW41 described below.

5.4.4 Complementation analysis with pTM1, pTM2, and pSW41

Figure 5.8 shows a schematic diagram of the transcomplementation systems that are described below. Plasmid pTM2 was transformed into *E.coli* JA221 containing pTM1 and assayed under non-inducing, inducing and repressing conditions in the absence of IPTG. pSW41 was also transformed into *E.coli* JA221 containing pTM1 and pTM2 and the presence of all three plasmids was verified by restriction digests of plasmid DNA isolated from the transformants. JA221 pTM1 pTM2 pSW41 was assayed under non-inducing, inducing and repressing conditions in the presence and absence of IPTG. The results of all these transcomplementation assays are shown in Table 5.8.

JA221 pTM1 pTM2 shows high constitutive amidase expression with an average specific activity of 33.2 units (line 1). This expression is independent of IPTG and is similar to the levels of expression seen with pTM1 and pSW24. The presence of pSW41 causes a 36 fold reduction in amidase expression from pTM1, pTM2 in the absence of IPTG, (line 2), and a 27 fold reduction in the presence of IPTG (line 3). In some of the assays with pTM1, pTM2 and pSW41 grown in the presence of IPTG, there appeared to be relief of AmiC repression, this is probably due the unstable production of AmiC from pSW41 as described in section 5.2.2.

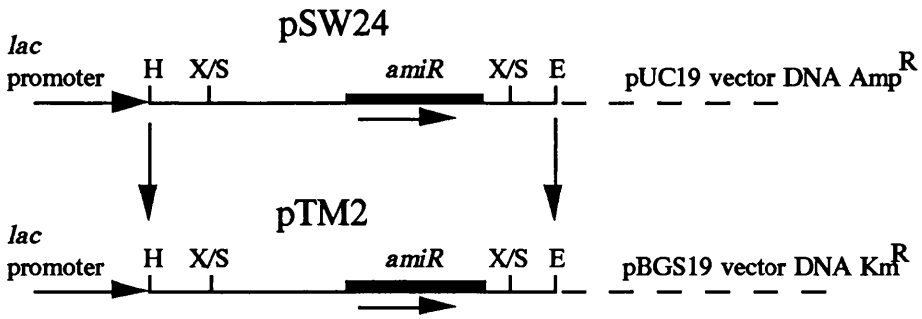


Figure 5.7. Construction of pTM2 and its restriction map. The 1.5kb *Hind* III- *Eco* RI fragment from pSW24 was subcloned into plasmid pBGS19-. Abbreviations are as follows: H= *Hind* III, E= *Eco* RI, X/S = hybrid site generated by ligation of *Xho* I and *Sal* I restriction targets.

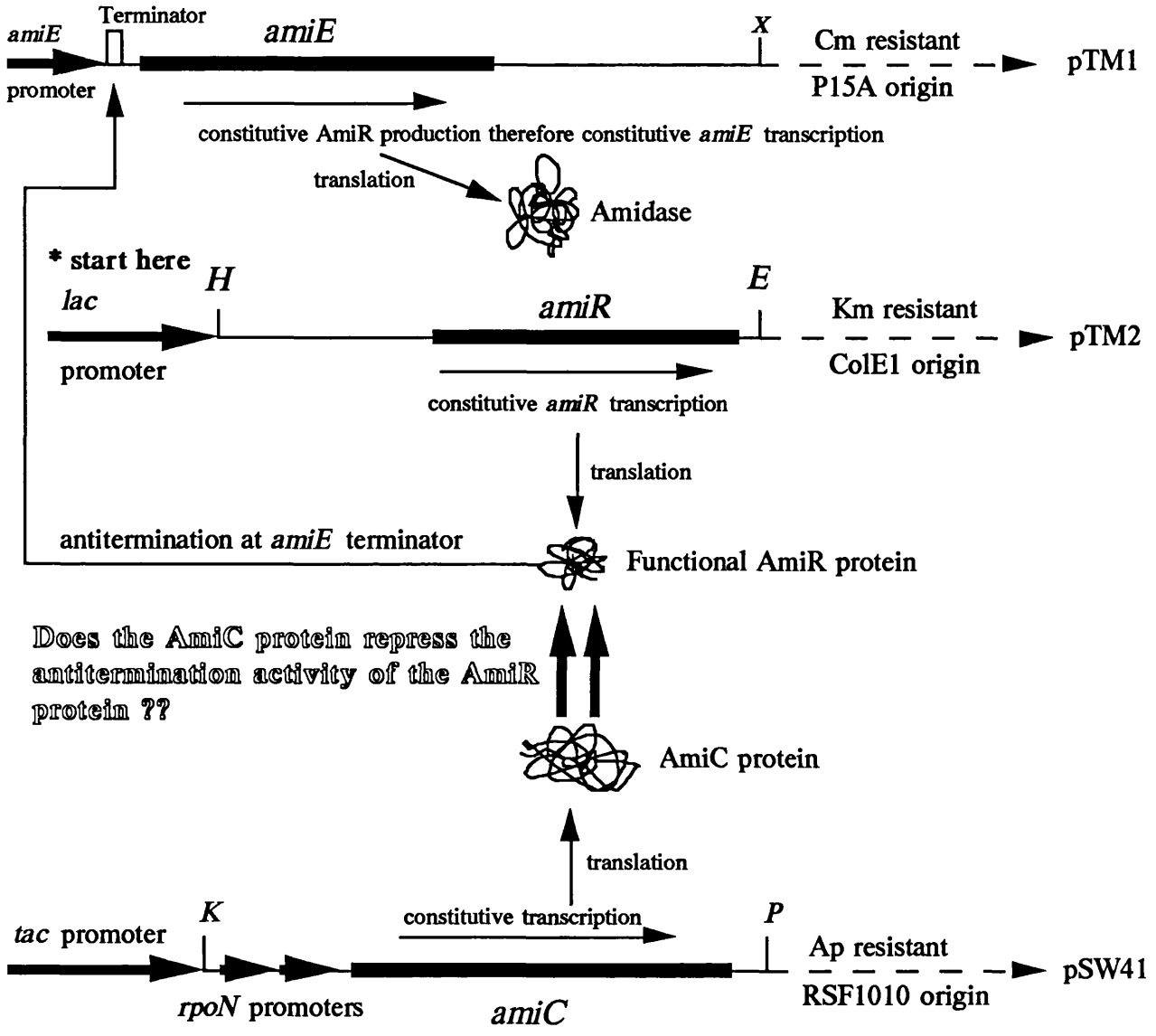


Figure 5.8. A schematic diagram showing the transcomplementation system used to test whether AmiC represses the antitermination activity of AmiR by a protein-protein interaction. pTM2 produces AmiR constitutively from the *lac promoter* and pSW41 produces AmiC constitutively from the *tac* promoter. pTM1 only produces amidase (which is assayed) in the presence of functional AmiR.

TABLE 5.8. Amidase activities[a] from *E.coli* strain JA221 carrying plasmids pTM1, pTM2 and pSW41.

Plasmids	IPTG	Growth Conditions				
		Glucose	Glucose/ Lactamide	Succinate	Succinate/ Lactamide	Succinate/ Butyramide
pTM1/pTM2	-	30.8	31.8	31.0	33.6	39.1
pTM1/pTM2/ pSW41	-	0.9	1.0	0.9	0.9	0.9
pTM1/pTM2/ pSW41	+	1.1	1.3	5.5	30.3	1.3

a) Amidase activity was measured as μmoles acetohydroxamate produced per minute per mg bacteria. The values presented are the means of duplicate assays carried out on at least three separate occasions.

Since the introduction of pSW41 into JA221 containing pTM1 and pTM2 causes repression of amidase activity this strongly suggests that AmiC represses AmiR activity by a protein-protein interaction. This repression could be by a direct protein-protein interaction, alternatively AmiC might covalently modify AmiR by phosphorylation, in a manner analogous to the repression of BglG activity by BglF (Amster–Choder *et al*, 1989). The repression of AmiR by AmiC in this transcomplementation system was not relieved by amide inducer, a similar result to that found when pSW41 was introduced in *P.aeruginosa* mutants which produce amidase constitutively. This could be because insufficient inducer is entering the cells.

5.5 Does AmiC have protein kinase activity?

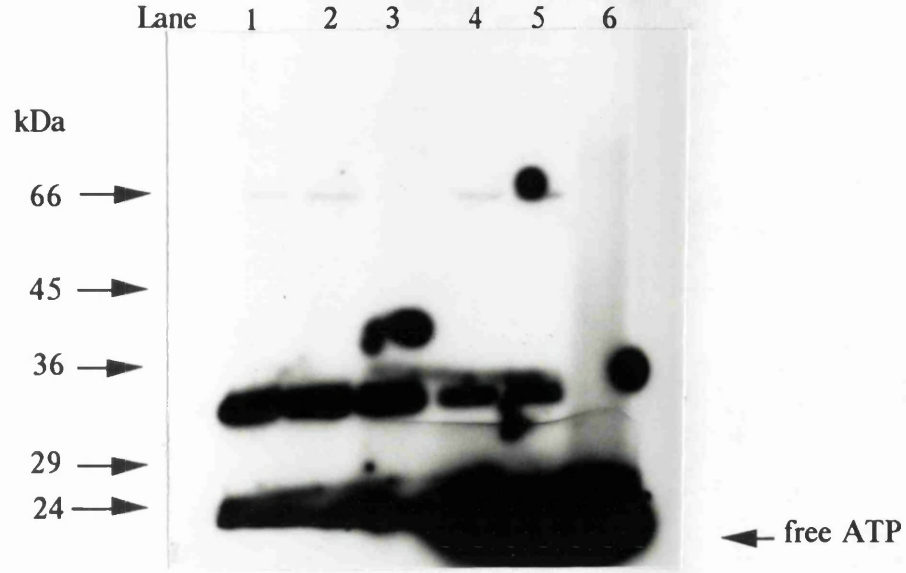
Though the experiment above has indicated that AmiC repression of AmiR occurs at the protein level it has not distinguished between the direct interaction, or covalent modification hypotheses. Two bacterial catabolic operons are known (*bgl* and *sac*, see Chapter one) where transcription antitermination is involved in the induction process, and in both these operons there is a negative regulator which repress the antiterminator. With the *bgl* operon, this repression is mediated by protein phosphorylation (Amster-Choder, 1989). Although there is no homology between the regulators involved in these operons and *amiC* and *amiR*, it was decided to investigate whether AmiC had protein kinase activity and might repress the antitermination activity of AmiR by a phosphorylation reaction.

5.5.1 Analysis of AmiC protein kinase activity

To investigate AmiC phosphorylation of AmiR *in vitro*, purified AmiC was incubated with a variety of crude extracts in the presence of γ-^{32}P—ATP as described in Chapter two. Following the phosphorylation reaction the samples were analysed on a 10% SDS-PAGE gel. The autoradiograph obtained from the first protein kinase assay gel is shown in Figure 5.9.

Lane 1 contains a crude extract of the amidase deletion strain PAC452, incubated with purified AmiC. In this lane there are two bands representing phosphorylated proteins. The major band has a molecular weight of approximately 34kDa and the minor band has a Mwt of 66kDa. Lane two contains a crude extract from PAC452 containing the *amiR* expression vector pSW40 grown in the absence of IPTG with purified AmiC. This crude extract should only contain small amounts of AmiR. Again two phosphorylated protein bands are seen. Unfortunately the resolution of proteins around the molecular weight of AmiR (23kDa) is very poor and it is not possible to identify an additional band which might correspond to phosphorylated AmiR. Lane 3 contains PAC452 pSW40 grown in the presence of IPTG but was incubated in the absence of purified AmiC. The major band at 34kDa is still present but interestingly the

PART A



PART B

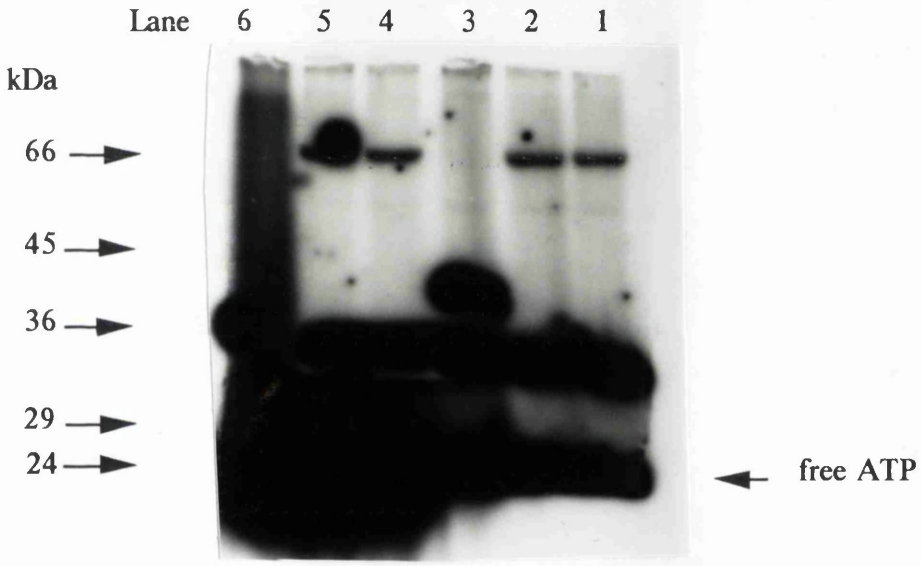


Figure 5.9. Protein kinase assay gel using purified AmiC and crude cell extracts. Part A shows a six hour exposure of the gel and part B shows an overnight exposure of the same gel. The dark circles of radioactivity represent free ATP spilt on the gel during transfer to Whatmann 3MM papaer. Samples were run on 10% SDS-PAGE gel and the positions of molecular weight standards are shown down the side of each photograph. The contents of each lane are as follows: Lane 1, crude extract of PAC452 + AmiC; Lane 2, crude extract of PAC452 pSW40 + AmiC; Lane 3, crude extract of PAC452 pSW40 + IPTG; Lane 4, crude extract of PAC452 pSW40 + IPTG + AmiC; Lane 5, crude extract of PAC452 pSW40 + IPTG + AmiC + lactamide; Lane 6, purified AmiC alone.

66kDa protein does not appear to have been phosphorylated, in the absence of AmiC. Lane 4 contains an identical crude extract to lane 3 except AmiC was added to the reaction and once again the 66kDa protein is phosphorylated. Lane 5 also contains PAC452 pSW40 + IPTG crude extract incubated with AmiC and lactamide. The 66KDa protein is phosphorylated under these conditions. Lane 6 contains purified AmiC incubated with γ-^{32}P-ATP and shows no evidence of a phosphorylated protein band.

These results do not provide any evidence for AmiC phosphorylating AmiR, principally because the gel did not resolve the proteins around the molecular weight of AmiR. However this gel provides clear evidence for the AmiC dependent phosphorylation of a 66kDa protein. The phosphorylation process appears to work in the presence and absence of amide inducer which suggests this might be a relaxed specificity phosphorylation by AmiC. The identity of this 66kDa protein is completely unknown but since it is present in PAC452, the amidase deletion strain, this strongly suggests the gene for this protein is unlinked to the amidase genes. Since lane six, only containing AmiC + γ-^{32}P-ATP showed no evidence of phosphorylated AmiC, this implies that AmiC is not capable of autophosphorylation. This result does not preclude the possibility that AmiC might bind ATP, since the gel was run under denaturing conditions. The major phosphorylated protein band at 34kDa presumably represents a protein which is being phosphorylated by a protein kinase present in the crude extracts.

A second experiment was carried out where the samples were analysed on a 15% SDS-PAGE gel, in an attempt to resolve the lower molecular weight proteins and the results are shown in Figure 5.10. This gel has resolved proteins of a lower molecular weight and several additional bands are seen. However it is not possible to distinguish the band patterns in crude extracts with or without AmiR, so once again this gel does not provide any evidence for the phosphorylation of AmiR by AmiC. However, this gel does again show the

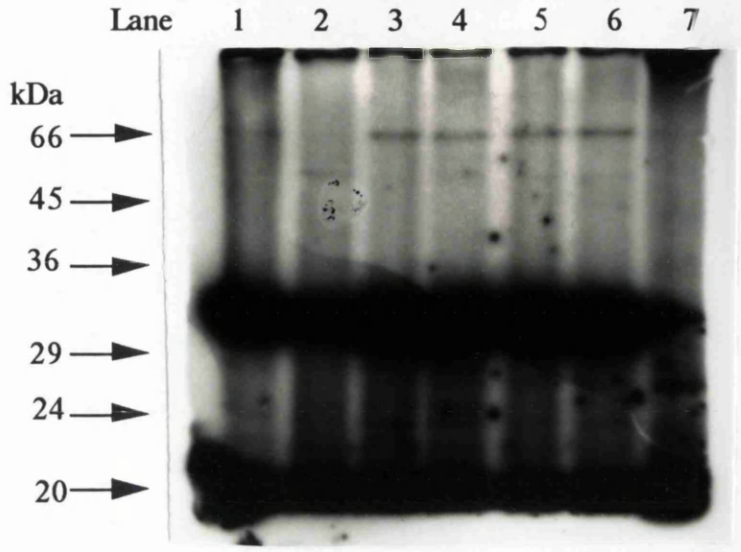


Figure 5.10. Protein kinase assay gel using purified AmiC and crude cell extracts. Samples were run on a 15% SDS-PAGE gel and the positions of molecular weight standards are shown. Samples were loaded as follows: Lane 1, crude extract of PAC452 + AmiC; lane 2, crude extract of PAC452 pSW40 + IPTG; lane 3, crude extract of PAC452 pSW40 + IPTG + AmiC; lane 4, crude extract of PAC452 pSw40 + IPTG + 2x normal amount of AmiC; lane 5 + lane 6, identical to lane 3; lane 7, crude extract of *E.coli* strain JM101 + AmiC.

AmiC dependent phosphorylation of the 66kDa protein seen in the last gel. In lane 4 twice as much AmiC was added to the AmiR containing crude extract compared to lane 3. The intensity of the 66kDa band in both lanes is the same which indicates that in the phosphorylation reaction AmiC was not limiting. The crude extract from *E.coli* (lane 7) does not show the phosphorylation of the 66kDa protein, but does show phosphorylation of two major protein bands with approximate molecular weights of 26kDa and 34kDa.

These results would suggest that AmiC has protein kinase activity and can transfer phosphate from ATP to a 66kDa protein present in *P.aeruginosa*. This transfer is not effected by the presence of inducer and could be a non-specific reaction. The possibility that another protein present in the crude cell extracts is charging AmiC with phosphate and AmiC is not directly transferring phosphate from ATP to the 66kDa protein seems unlikely since no radioactive band was detected with the molecular weight of AmiC. The phosphorylation of AmiR has not been demonstrated. This could be because AmiR might be present in small quantities in the cell, indeed there is no evidence of AmiR production from pSW40 on an SDS-PAGE gel. Also there is a large amount of background on these gels perhaps because a large amount of free ATP is present in the reactions and faint bands corresponding to AmiR would be indistinguishable from the background.

This problem could have been alleviated by running the reaction mixtures down size exclusion columns to remove unincoporated ATP. Secondly, the majority of radioactive bands produced in these reactions correspond to protein kinases present in the crude extracts, acting on their substrates. These radioactive proteins make identification of an AmiR band very difficult. To alleviate this problem it should be possible to incubate the crude extract in the presence of cold ATP, followed by radioactive ATP and AmiC. This should reduce the number of bands on the gel, and allow

easier AmiR identification. Thirdly, the reaction conditions might not be optimal for AmiR phosphorylation, e.g incubation time, pH, salt concentration etc. Ideally to show AmiC phosphorylation of AmiR, one would use purified AmiR and AmiC. Unfortunately, insufficient time was available to optimise these experiments and employ the modifications described above.

5.6 Summary

The complementation analysis with the *amiR* expression vector pSW40, provided several interesting results. Firstly, it would appear that saturating levels of AmiR can easily be provided by pSW40, and yet no major protein is seen by SDS-PAGE analysis (Chapter four). This would imply that only small amounts of AmiR are required for the antitermination process. Secondly the over production of *amiR* by pSW40 in PAC1 leads to constitutive amidase expression. At first this result was thought to be because *amiR* expression was not from its normal promoter, but later results in this chapter indicated AmiC regulated AmiR activity by a protein-protein interaction. PAC1 contains a wild type *amiC* gene and yet cannot repress the AmiR produced by pSW40. This would suggest there is some level of stoichiometry between the amounts of AmiR and AmiC in a normal situation.

The introduction of the *amiC* expression vector into three amidase constitutive strains of *P.aeruginosa* showed repression of amidase synthesis. This confirmed the strains were *amiC* $^-$ and also showed that *amiC* was a negative regulator of amidase expression. Interestingly *P.aeruginosa* strains containing pSW41 were non-inducible. This could be an inducer permeation problem, since there does not appear to be an active transport mechanism for amides in *P.aeruginosa* (Farin, 1976). Also if there is some stoichiometry between the levels of AmiC and AmiR, then massive increases in the levels of AmiC with respect to AmiR could also render the system non-inducible particularly if amides have some difficulty getting into *P.aeruginosa*.

The preliminary dot blot analysis on RNA isolated from PAC452 pSW101 indicated that *amiCR* transcription was regulated and this lead to the hypothesis that *amiC* might form part of an autogeneous control circuit, regulating its

own expression and that of *amiR*. This model was tested by assaying the purified AmiC for DNA binding activity and by transcomplementation studies in *E.coli*. The results showed that AmiC did not bind DNA and this hypothesis was rejected. An alternative model for AmiC repression was proposed involving an interaction at the protein level with AmiR. This model was tested using a three plasmid complementation system in *E.coli*, which confirmed that AmiC repressed the antitermination activity of AmiR by some form of protein-protein interaction. Attempts were made to show AmiC dependent phosphorylation of AmiR which were inconclusive, but they did show that AmiC could phosphorylate a 66kDa protein. In conclusion, these studies showed that AmiC had protein kinase activity and could potentially phosphorylate AmiR to repress its antitermination activity.

CHAPTER SIX - IDENTIFICATION OF *amiX* AND *amiY*, TRANSCRIPT ANALYSIS AND NITROGEN REGULATION OF THE AMIDASE GENES IN *E.coli*.

6.1 Introduction

The results from the previous chapters have identified a new regulatory gene *amiC*. The *amiC* protein product has been purified and N-terminally sequenced. The transcomplementation experiments described in Chapter five confirmed that AmiC was a negative regulator of amidase expression. In addition it was shown that AmiC repressed AmiR by some form of protein-protein interaction, however it was not shown unequivocally that AmiC repressed AmiR by a phosphorylation reaction, but this seems likely.

Two possible promoter elements were identified upstream of *amiC* which had homology to the consensus *ntrA* dependent promoter. The function of these promoters *in vivo* remains unclear though, since their removal still allowed *amiR* expression. Since the stop codon for *amiC* and the start codon for *amiR* overlap it was assumed that these two genes form part of a polycistronic operon, possibly transcribed from the *ntrA* dependent promoter elements.

At the level of protein function it is quite simple to build a model for how amidase induction might occur - AmiC would repress the antitermination activity of AmiR by a phosphorylation reaction, which in turn would be dependent on the presence or absence of inducer which would interact with AmiC regulating its activity.

At the transcriptional level though, it is very difficult to draw up a model. Promoter activity has been found to originate from an unidentified region of DNA upstream of the 658bp *Xho*I fragment, and two *ntrA* dependent promoter elements have been identified by sequence homology within the 658bp

*Xho*I fragment, which has also been shown to have promoter activity *in vivo*. But why are there two promoters? and are these promoters actually functional? Preliminary dot blot analysis described in Chapter five showed that *amiC* and *amiR* transcription is regulated in response to inducer. How this regulation occurs is as yet completely unknown. To address these questions it was decided to investigate the transcription of the amidase genes by *in vivo* and *in vitro* techniques. As a complement to this analysis the DNA sequence of the remaining gaps in the 5.3kb *Hin*dIII-*Sal*I fragment which had originally been cloned from PAC1 was to be determined.

6.2 Nitrogen Regulation of the amidase genes in *E.coli.*

The first stage in the investigation of the transcription of the amidase genes involved an analysis of the two potential *ntrA* dependent promoters upstream of *amiC*. A discussion of the regulation of *ntrA* (σ^{54}) dependent promoters is presented in Chapter one. In summary, all known promoters which are recognised by the sigma factor NtrA require an activator protein to function (Thony and Hennecke, 1989). In well characterised examples such as the *glnAp2* promoter of glutamine synthetase the activator protein, NR_I-phosphate (NtrC) stimulates closed to open complex formation of RNA polymerase at the promoter thus allowing transcriptional elongation (Popham *et al*, 1989).

It is tempting to speculate that the *ntrA* dependent promoters upstream of *amiC* are regulated by a global activator in *P.aeruginosa* which responds to nitrogen limitation, in a manner analogous to NtrC in enteric bacteria, since one of the products of amide hydrolysis by amidase is ammonia. Indeed Jansenn *et al* (1982a) have shown that amidase expression in PAO1 is activated under nitrogen limiting conditions. However, in PAC strains there does not appear to be activation of amidase expression under nitrogen limiting conditions and yet glutamine synthetase activities are elevated (Potts and Clarke, 1976; Nyberg and Clarke, 1978). This indicates that in PAC strains the global nitrogen regulation system is intact and functional, but apparently fails to function on the amidase genes. This could be due to a mutation in the amidase locus during the selection for a "wild" type

strain with high (but inducible) levels of amidase activity, which was initially carried out to isolate PAC1 (Kelly, 1962). Therefore the nature of the activator which normally regulates these two promoters, if indeed they function, remains unclear, it could be a component of the global nitrogen regulatory system, or some other gene.

Activators of *ntrA* dependent promoters have conserved domains and can be interchanged (Stock *et al,* 1989). Dixon (1986) showed that the *xyl*CAB promoter could be heterologously activated by *ntrC, nifA* and the normal activator *xylR.* Consequently it was decided to test whether these two *ntrA* promoters were responsible for the promoter activity known to be present in the 658bp *Xho*I fragment, by trying to activate them with the nitrogen regulatory gene *ntrC in E.coli.*

6.2.1 Amidase activity from pJB950 and pAS20 in JA221 under nitrogen limiting conditions.

In order to demonstrate *ntrC* activation of the *ntrA* dependent promoters, *E.coli* JA221 containing plasmids pAS20 or pJB950 were grown under nitrogen limiting conditions, which causes activation and increased expression of the *ntrC* gene product NR_I, and amidase assayed. The results are shown in Table 6.1 Plasmid pJB950 normally produces a low level of amidase constitutively (4 units) (Cousens *et al,* 1987). Under nitrogen limiting conditions there is a ten fold increase in amidase expression with an average specific activity of 38 units and there appears to be a further increase in amidase activity in the presence of lactamide. Plasmid pAS20 shows extremely low amidase activity when grown in nitrogen excess conditions. However, under nitrogen limiting conditions pAS20 shows an inducible phenotype, with an average induced specific activity of 5.6 units. This is the first result showing inducible amidase activity in *E.coli.* This suggests that all the genes required for inducible amidase expression are present on the 5.3kb *Hin*dIII-*Sal*I *Pseudomonas* DNA fragment of pAS20, but the induced expression is only detected when there is an increase in transcription of *amiC* and *amiR.*

TABLE 6.1. Amidase activities[a] from pAS20 and pJB950 under nitrogen limiting conditions in *E.coli* strain JA221.

		Growth Conditions					
Plasmid	N	Glucose	Glucose/ Lactamide	Succinate	Succinate/ Lactamide	Succinate/ Butyramide	Mean
pAS20	+	0.0	0.1	0.0	0.0	0.0	-
pAS20	-	0.0	6.2	0.4	4.9	0.1	-
pJB950	+	3.8	4.0	3.2	3.8	3.5	3.7
pJB950	-	21.6	43.8	24.7	61.0	38.6	38.0

a) Amidase activity was measured as μmoles acetohydroxamate produced per minute per mg bacteria. Values presented are the mean values of duplicate assays carried out on at least three separate occasions. The column labelled "N" refers to nitrogen excess (+) and nitrogen limiting (-) growth conditions.

To confirm that the increased amidase activity was a result of transcriptional activation of the two *ntrA* dependent promoter elements a selection of plasmids with and without these promoters were assayed under nitrogen limiting conditions as described below.

6.2.2 Amidase activity from pSW2, pSW3, pSW5 and pSW36, in JA221 under nitrogen limiting conditions

Amidase activity was measured from plasmids pSW2, pSW3 and pSW5 in *E.coli* JA221 under nitrogen limiting conditions and the results are shown in Table 6.2.

Table 6.2 - Amidase activities[a] from pSW2, pSW3, pSW5 and pSW36 in *E.coli* JA221 under nitrogen limiting conditions.

	Growth Conditions		
Plasmid	Nitrogen	Glucose	Succinate
pSW2	+		0.0
pSW2	-		0.0
pSW3	+		2.0
pSW3	-		2.1
pSW5	+		10.5
pSW5	-		8.7
pSW36	+	1.5	1.3
pSW36	-	4.0	3.0

a) Amidase activity was measured as μmoles acetohydroxamate produced per minute per mg bacteria. Values presented are the mean of duplicate assays carried out on at least three separate occasions. The column labelled "nitrogen" refers to nitrogen excess and nitrogen limiting growth conditions.

pSW2 (derived from pJB950) has the 658bp *Xho*I fragment containing the *ntrA* dependent promoters deleted and has the 1.5kb *Xho*I *amiR* fragment inverted. This plasmid shows low background amidase expression under nitrogen excess growth conditions (see Chapter 3). There is no activation of amidase expression from this plasmid under nitrogen limiting conditions.

Plasmid pSW3, a derivative of pAS20 has the 658bp *Xho*I fragment missing, but the 1.5kb *Xho*I *amiR* fragment in the normal orientation. Under nitrogen excess growth conditions this plasmid shows constitutive amidase expression of approximately 2 units (see Chapter 3). Under nitrogen limiting

conditions this plasmid shows an amidase activity of 2.1 units, which is similar to the level seen under nitrogen excess. This result confirms that the *ntrA* dependent promoter elements present in the 658bp *Xho*I fragment are required for nitrogen activation of the amidase genes in *E.coli*. This result also suggests that the unidentified upstream promoter element which is allowing *amiR* expression when the *ntrA* dependent promoters are deleted, is not capable of being activated under nitrogen limiting conditions. A similar result is obtained with pSW5 (pJB950 homologue of pSW3) which also shows no nitrogen activation.

To confirm that *amiC* was not involved in the nitrogen activation effect, plasmid pSW36 which has a 250bp deletion within the *amiC* gene was assayed under nitrogen limiting conditions and the results are shown in Table 6.2. This plasmid does show nitrogen activation, confirming that *amiC* is not involved in this effect.

To investigate the nitrogen activation of the amidase genes in *E.coli* more thoroughly an isogenic series of *E.coli* strains with mutation in the nitrogen regulatory genes were obtained from R. Dixon (AFRC Nitrogen Fixation Unit, University of Sussex). A cloned *ntrC* gene from *Klebsiella pneumoniae* was also obtained for transcomplementation experiments.

6.2.3 Amidase activity from pJB950 and pAS20 in *E.coli* strains with mutations in *ntrA* and *ntrC*, and complementation with plasmid encoded *ntrC*.

The wild type strain used for these experiments was strain ET8000 (*ntrA*$^+$, *B*$^+$, *C*$^+$). Plasmids pAS20 and pJB950 were transformed into this strain and amidase activity measured under nitrogen excess and nitrogen limiting conditions. The results are shown in Table 6.3. Plasmid pAS20 shows background levels of amidase activity under nitrogen excess conditions and an inducible phenotype under conditions of nitrogen limitation. The average induced level of amidase activity is 1.7 units. Plasmid pJB950 also shows activation under nitrogen limiting conditions, although the level of activation is only

Table 6.3 - Amidase activities from plasmids pAS20 and pJB950 in *E.coli* strain ET8000.

Plasmid	Nitrogen	Growth Conditions				
		Glucose	Glucose/ Lactamide	Succinate	Succinate/ Lactamide	Mean
pAS20	+		0.3	0.0	0.1	-
pAS20	-		1.1	0.0	2.6	-
pJB950	+	3.2	-	1.4	-	2.3
pJB950	-	8.2	-	3.6	-	5.9

a) Amidase activity was measured as μmoles acetohydroxamate produced per minute per mg bacteria. Values presented are the mean of duplicate assays carried out on at least three separate occasions. The column labelled "nitrogen" refers to nitrogen excess and nitrogen limiting growth conditions.

three fold, which is considerably less than that seen with *E.coli* strain JA221.

To test whether *ntrC* was responsible for the nitrogen activation of amidase in *E.coli* plasmid pJB950 was transformed into the *ntrC*$^-$ strain ET8556 and amidase assayed in the presence and absence of nitrogen. The results are shown in Table 6.4 . Amidase was assayed with succinate as the carbon source and the average activity seen with nitrogen present was 4.8 units. Under nitrogen limitation there was a slight increase in amidase activity, to an average value of 6.0 units, but this is not considered significant. This result confirms that the activation of amidase expression under nitrogen limiting conditions in *E.coli* is an *ntrC* mediated effect.

TABLE 6.4 - Amidase activites[a] from plasmid pJB950 in the *ntrC*⁻ strain ET8556 and complementation with pMM14

Strain	Plasmid	Growth Conditions			
		Nitrogen	Glucose	Succinate	Mean
ET8556	pJB950	+		4.8	
ET8556	pJB950	-		6.0	
ET8556	pJB950/pMM14	+	20.4	18.0	19.2
ET8556	pJB950/pMM14	-	21.6	13.4	17.5
JA221	pJB950	+	-	3.2	-
JA221	pJB950	-	-	24.7	-
JA221	pJB950/pMM14	+	-	23.5	-
JA221	pJB950/pMM14	-	-	26.9	-

a) Amidase activity was measured as μmoles acetohydroxamate produced per minute per mg bacteria. Values presented are the mean of duplicate assays carried out on at least three separate occasions. The column labelled "Nitrogen" refers to growth under nitrogen excess or nitrogen limiting conditions.

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A cloned *ntrC* gene on plasmid pMM14 was transformed into ET8556 containing plasmid pJB950 to complement the chromosomal mutation in *ntrC*. The *ntrC* gene present on plasmid pMM14 is a mutant gene which no longer requires *ntrB* activation for it to function (Merrick, 1983), therefore it will activate transcription in the absence and presence of nitrogen. Amidase activity was measured from ET8556 containing pJB950 and pMM14, with and without nitrogen and the results are shown in Table 6.4. In the presence of nitrogen an average amidase activity of 19.2 units is seen. This level is three times higher than that seen in the

absence of *ntrC*. A similar result is obtained under nitrogen limiting conditions, with a two fold increase in amidase expression, compared to the pMM14 minus strain . When plasmid pMM14 was transformed into *E.coli* JA221 pJB950 there was also an increase in amidase expression even under nitrogen excess growth conditions (see Table 6.4). These results confirm that in the presence of *ntrC* a marked increase in amidase activity occurs which is dependent on sequences present in the 658bp *Xho*I fragment.

Since this activation of *amiCR* transcription is likely to be dependent on the two *ntrA* dependent promoter elements it was decided to investigate amidase in the *ntrA*$^-$ strain ET8045. It was expected that this strain would show background levels of amidase activity, since the two promoters would not function allowing *amiR* expression and there should be no activation under nitrogen limiting conditions. Plasmids pAS20 and pJB950 were transformed into ET8045 and amidase assayed under nitrogen limiting and nitrogen excess growth conditions. The results are shown in Table 6.5.

Surprisingly, both pAS20 and pJB950 show an increase in amidase activity, under nitrogen limitation and nitrogen excess conditions compared to the isogenic wild type strain ET8000 grown in nitrogen excess conditions. In strain ET8000 , pAS20 shows a very low induced level of amidase (average 0.15 units) when grown in the presence of excess nitrogen, whereas in the *ntrA*$^-$ strain (ET8045) pAS20 shows an inducible phenotype, but the induced amidase activity has risen to 1.2 units. This level of expression is similar to the level of activity seen when ET8000 pAS20 is grown under nitrogen limiting conditions (average induced level is 1.7 units). There is a further two fold increase in the amidase activity seen from ET8045 pAS20 when this strain is grown under nitrogen limiting conditions.

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TABLE 6.5 - Amidase activities[a] from plasmids pAS20 and pJB950 in the *ntrA*⁻ strain ET8045.

Plasmid	Nitrogen	Growth conditions			
		Glucose	Succinate	Succinate/ Lactamide	Mean
pAS20	+	-	0.0	1.2	-
pAS20	-	-	0.3	2.6	-
pJB950	+	17.2	11.3	-	14.3
pJB950	-	16.0	10.4	-	13.2

a) Amidase activity was measured as μmoles acetohydroxamate produced per minute per mg bacteria. Values presented are the mean of duplicate assays carried out on at least three separate occasions. The column labelled "Nitrogen" refers to growth under nitrogen excess and nitrogen limiting conditions.

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This result is somewhat surprising since *ntrA* would be expected to be required for activated transcription from the *ntrA* dependent promoter elements within the 658bp *Xho*I fragment. pJB950 also shows increased amidase expression in strain ET8045 with an average activity under nitrogen excess growth conditions of 14.3 units. This level of expression is seven fold higher than that seen in the isogenic wild type strain ET8000 grown in nitrogen excess conditions, but only two fold higher than the level seen when ET8000 pJB950 is nitrogen activated. There is no increase in amidase activity when ET8045 pJB950 is grown under nitrogen limiting conditions.

In theory, the absence of *ntrA* automatically means that no transcription can occur from the two potential *ntrA* dependent promoters upstream of *amiC*.

Yet the level of expression is actually similar to that seen under nitrogen limiting conditions when *ntrA* is present. The increased level of expression seen in an *ntrA*$^-$ background is similar to the increase seen when the 658bp fragment is deleted from pAS20 and pJB950. Clearly, *amiR* expression in ET8045 must be originating from a promoter which is not *ntrA* dependent (for example the *amiE* promoter?) and this is probably the same promoter that is used to drive *amiR* expression when the 658bp *Xho*I fragment is deleted. This would explain why both situations give similar levels of amidase activity.

The results above suggest that there is another promoter upstream of the 658bp *Xho*I fragment which is stronger than the *ntrA* dependent promoters in this fragment and this promoter can be used for *amiC/R* transcription. More importantly though, it would appear that *ntrA* can play a role in regulating expression from this upstream promoter as a negative controlling element, since removal of *ntrA* from the host leads to higher levels of amidase. Removal of *ntrA* from the system does not appear to effect the inducibility of amidase expression, it simply increases the induced levels. It was decided to investigate whether a similar effect was seen in *ntrA* mutants of *P.aeruginosa* and this is described below.

6.3 Amidase activity in *ntrA* mutants of *P.aeruginosa*

The *ntrA* gene from *P.aeruginosa* had recently been cloned and this gene was used to construct a mutant strain that was insertionally inactivated in its chromosomal *ntrA* gene (Ishimoto *et al*, 1989). Strain PAK-N1 was obtained as well as the isogenic wild type strain and amidase expression from the two strains was investigated.

6.3.1 Amidase activity from *P.aeruginosa* strain PAK-SR and the isogenic *ntrA*$^-$ strain, PAK-N1.

Strain PAK-SR is a streptomycin resistant mutant of the wild type *P. aeruginosa* strain PAK and has a wild type *ntrA* gene. An *ntrA*$^-$ strain was constructed from PAK-SR by gene replacement using a cloned *ntrA* gene con-

taining a tetracycline resistance gene insertion (Ishimoto and Lory, 1989). The wild type strain PAK-SR and the isogenic *ntrA*$^-$ mutant strain PAK-N1 were assayed for amidase activity and the results are shown in Table 6.6.

TABLE 6.6 - Amidase activites[a] from *P.aeruginosa* strains PAK and PAK-N1

	Growth conditions				
Strain	Succinate	Succinate/ Lactamide	Lactate	Lactate/ Lactamide	Lactate/ Butyramide
PAK (*ntrA*$^+$)	0.1	0.5	0.2	0.7	0.1
PAK-N1 (*ntrA*$^-$)	0.3	21.7	0.4	14.6	0.7

a) Amidase activity was measured as μmoles acetohydroxamate produced per minute per mg bacteria. Values presented are the mean of duplicate assays carried out on at least three separate occasions.

Strain PAK-SR shows an inducible phenotype but the induced level of amidase is very low (average induced activity = 0.6 units). The *ntrA*$^-$ strain PAK-N1 also shows an inducible phenotype but there is an increase in the average induced amidase levels, to 18.2 units. This represents a 36 fold increase in amidase expression, simply by removing the sigma factor NtrA. This result provides further evidence for *ntrA* (σ^{54}) playing a role in down-regulating amidase expression. Since the wild type strain PAK-SR gave very low levels of amidase compared with PAC1 it was decided to investigate amidase expression in an *ntrA* mutant of PAC1, to test whether a similar effect could be produced.

6.3.2 Construction of an *ntrA*⁻ mutant of *P.aeruginosa* strain PAC1 and measurements of amidase activity.

An *ntrA*⁻ mutant was constructed using plasmid pKI11, in a manner similar to that used by Ishimoto and Lory (1989). pKI11 contains a cloned *ntrA* gene insertionally inactivated by having a tetracycline resistance gene. This plasmid has a ColE1 origin of replication therefore it will not replicate in *P.aeruginosa*. The plasmid also contains the *mob* region from pRP4 so the plasmid can be mobilised into *P.aeruginosa*. Therefore pKI11 is a suicide plasmid which can be introduced into *P.aeruginosa* by mobilisation but cannot replicate once inside the host. When this plasmid is introduced into *P.aeruginosa* homologous recombination events can occur between the chromosomal *ntrA* gene and the mutant tetracycline resistant *ntrA* gene on the plasmid. Therefore by selecting transconjugants which are tetracycline resistant, one automatically selects for strains where recombination has occurred between the plasmid and the chromosome, rendering the chromosomal *ntrA* gene inactive, and the strain tetracycline resistant. The suicide plasmid also encodes a gene for carbenicillin resistance and if only one cross-over event occurs (thus retaining a wild type chromosomal *ntrA* gene), the strain will be tetracycline resistant and carbenicillin resistant, since the whole vector will have been inserted in the chromosome. So to isolate a strain in which a double cross-over event has occurred and the *ntrA* gene has been inactivated, strains are screened for sensitivity to carbenicillin and resistance to tetracycline.

Plasmid pKI11 was mobilised into the wild type strain PAC1 and transconjugants were selected for on tetracycline plates. Only one transconjugant was obtained and fortuitously this strain was carbenicillin sensitive indicating that a double cross-over event had occurred and the tetracycline resistance gene had been inserted into the chromosome within the *ntrA* gene. This strain was named PAC200.

The pilin gene of *P.aeruginosa* is transcribed from an *ntrA* dependent

promoter and this protein is required for flagella formation. Consequently an *ntrA*$^-$ strain of *P.aeruginosa* is non-motile (Ishimoto *et al*, 1989). To confirm that PAC200 was *ntrA*$^-$, exponentially growing cultures of PAC1 and PAC200 were examined with a phase contrast microscope and it was found that PAC200 was non-motile.

As final confirmation that the *ntrA* gene had been insertionally inactivated a Southern blot was carried out on PAC200 chromosomal DNA and probed with the insertionally inactivated *ntrA* gene in pKI11 (Ishimoto and Lory, 1989) as follows: chromosomal DNA was prepared from PAC1 and PAC200 and 5μg of each DNA was digested with *Xho*I and run on a 1% agarose gel with a λ/*Hin*dIII size marker. Following electrophoresis the DNA was transferred from the gel to a nylon membrane by the alkali blot procedure. The Southern blot was subsequently probed with the 5.7kb *Xho*I fragment from pKI11 which encompasses the insertionally activated *ntrA* gene (See Figure 6.1). 50ng of this fragment was ^{32}P labelled by random oligonucleotide priming and 1ng of λ DNA was included in the labelling reaction to hybridise with the λ/*Hin*dIII digest on the blot. The results are shown in Figure 6.1.

When *P.aeruginosa* strain PAK is digested with *Xho*I and probed with an *ntrA* gene, a single band is seen corresponding to an *Xho*I fragment of 4.3kb. In the *ntrA* mutant strain the size of this band increases to 5.7kb since the 1.4kb *tet* cassette has been introduced into the *ntrA* gene (Ishimoto and Lory, 1989). The results shown in Figure 6.1 indicate that in PAC1 a strong band is seen at 4.3kb but there is also a fainter band with a predicted size of 1.2kb. The source of this second band is unclear, but it could represent a difference in the restriction sites around the *ntrA* gene. In PAC200 the 4.3kb band has increased in size to 5.7kb as expected. Once again there is a fainter band which has a predicted molecular weight of 1.4kb. Since the major band at 4.3kb from PAC1 has increased in size by the appropriate amount in PAC200 this confirms that the *ntrA* gene has been insertionally inactivated. The reason for the additional low molecular weight bands seen with PAC1 and PAC200 DNA cannot be explained by differences in the

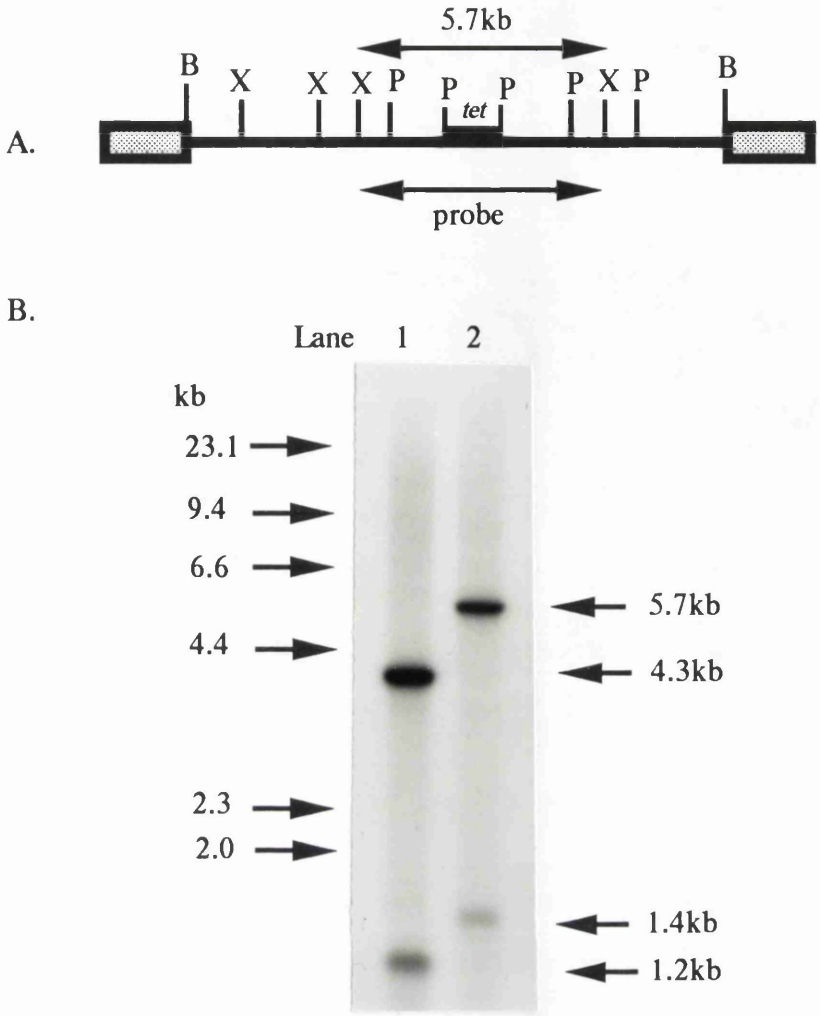


Figure 6.1. Southern hybridisation analysis of *P. aeruginosa* strains PAC1 and PAC200 using a mutant *ntrA* gene probe. Part A. Partial restriction map of plasmid pKI11, showing the location of the *Xho* I fragment used as a probe for part B. The native *Xho* I fragment encompassing the *ntrA* gene has a size of 4.3kb. The insertionally activated *ntrA* gene *Xho* I fragment, used here, has a size of 5.7kb. The location of the *tet* cassette used to inactivate the *ntrA* gene is shown. The boxes indicate pUC18 vector sequences. Abbreviations for restriction targets are as follows: B= *Bam* HI, X= *Xho* I, P= *Pst* I. Part B. Southern hybridisation of *XhoI* digested chromosomal DNA isolated from PAC1 and PAC200, with the 5.7kb *Xho* I fragment shown in part A . Lane 1 contains PAC1 DNA and shows two bands of 4.3kb and 1.2kb. Lane 2 contains DNA from PAC200 and shows two bands of 5.7kb and 1.4kb.

restriction maps of PAK and PAC strains at this locus, since the insertion of the *tet* cassette should only lead to the increase in size of one of the bands, as the *tet* cassette itself does not contain any *Xho*I restriction targets. Therefore it would appear that these faint bands represent non-specific hybridisation of the probe. The *ntrA*$^-$ strain PAC200 was assayed for amidase activity under non-inducing, inducing and repressing conditions and the results are shown in Table 6.7 along with the activities for PAC1 grown under the same conditions.

##########

TABLE 6.7 - Amidase activities[a] from *P.aeruginosa* strains PAC1 and the *ntrA*$^-$ mutant PAC200.

	Growth Conditions				
Strain	Succinate	Succinate/ Lactamide	Lactate	Lactate/ Lactamide	Lactate/ Butyramide
PAC1	0.1	8.1	0.1	2.5	0.0
PAC200	0.7	38.0	0.5	32.0	0.8

a) Amidase activity was measured as μmoles acetohydroxamate produced per minute per mg bacteria. Values shown are the mean of duplicate assays carried out on at least three separate occasions.

##########

PAC1 shows inducible amidase expression with an average induced activity of 5.3 units. PAC200 also shows an inducible activity but the average induced activity is now 35 units. Therefore the inactivation of *ntrA* causes a seven fold increase in amidase activity from PAC1. This result is similar to the results obtained with PAK-SR and PAK-N1 and confirms that *ntrA* is down regulating amidase expression in some way. However this damping effect does not appear to affect the inducibility of amidase expression.

These results indicate that the two *ntrA* promoters upstream of *amiC* are involved in the regulation of amidase expression and under the appropriate conditions can be activated by the nitrogen regulatory gene *ntrC*. Several results have indicated that *amiR* can be transcribed from a strong promoter upstream of the 658bp *Xho*I fragment (which contains the two *ntrA*-dependent promoters) and the results from this Chapter suggest that this promoter is not dependent on the sigma factor *ntrA*. In the absence of *ntrA* amidase activity remains inducible in *P.aeruginosa* but at a much higher level, suggesting *amiR* expression in this situation is originating from this upstream promoter. These would mean that *ntrA* is actually controlling transcription originating from this upstream promoter and can reduce it. To consider how this might occur it is necessary to look at the action of NtrA at *ntrA* dependent promoters.

ntrA dependent promoters fall into two categories defined as weak and strong (see Chapter one). These categories do not reflect strength of the promoters but the differences in σ^{54}-holoenzyme/promoter binding. With strong promoters, NtrA associates with RNA polymerase and forms a closed complex with the DNA. The closed complex is stable and can be detected by *in vitro* techniques (Popham *et al*, 1989). Activation of such promoters does not rely on the activator protein binding to an upstream DNA sequence, though maximal activation requires an upstream activator binding site, or high concentrations of activator. With weak *ntrA* dependent promoters the σ^{54}-holoenzyme closed complex cannot be readily detected and there is an absolute requirement for an upstream binding site for the activator protein, to cause transcription initiation (Morett and Buck, 1989).

It is not possible to say which class the two *ntrA* dependent promoters upstream of *amiC* fall into. However, if either promoter sequence is capable of forming a stable closed complex with σ^{54}-holoenzyme, then this could explain the apparent increase in amidase expression in the absence of NtrA. Such a stable closed complex could block transcription from the stronger upstream promoter, thus reducing the level of *amiCR* expression and therefore the level of *amiE*

expression. In the absence of NtrA, RNA polymerase would not bind to the promoter elements and expression from the upstream promoter would progress unhindered through *amiC* and *amiR*. This hypothesis would also explain why increased amidase activity is seen when the 658bp *Xho*I fragment is deleted. This fragment does not appear to contain any obvious transcription terminators but it does contain the two *ntrA* dependent promoter sequences. When *ntrC* activates amidase expression this could be due to two effects. Firstly, the closed complex at the promoter/(s) is being stimulated to form an open complex by *ntrC* thus allowing transcription from these promoters. Secondly, NtrC might simply destabalise the closed complex and not necessarily form productive open complexes, and this would allow transcription read through from the stronger upstream promoter. These hypotheses are tested later in this chapter by *in vitro* analysis of the transcripts from *ntrA*$^+$ and *ntrA*$^-$ strains of *E.coli*. Another possibility for the increased amidase expression in an *ntrA*$^-$ host could be that there is an unlinked gene, normally transcribed from an *ntrA* dependent promoter which represses amidase expression in some way, though this explanation seems unlikely since a similar effect is seen in *E.coli*.

6.4 Sequencing of the DNA between *amiE* and *amiC*- identification of *amiY*

Rehmat and Shapiro (1983) obtained mutator phage D3112 insertions which mapped between *amiE* and *amiC* and these were described as leaky mutants, i.e they were capable of growth on fluoroacetamide plates which selects for amidase negative strains, but they showed the ability to grow poorly on acetamide plates, indicating residual amidase activity. The attempts to identify a gene product encoded by this region with plasmid pSW42 failed and the transcomplementation experiments with pSW42 in PAC1 did not really provide any evidence for a regulatory gene encoded by this region of DNA. Yet the phage insertion results suggest

this region of DNA might be involved in regulating amidase expression. To try and identify any possible open reading frame within the region between *amiE* and *amiC* and to locate the promoter driving *amiR* expression in the absence of the 658bp *Xho*I fragment, it was decided to sequence this region of DNA from PAC1.

6.4.1 Construction of M13 clones and sequencing strategy

The sequencing strategy used is shown in Figure 6.2 along with the names of the clones that were constructed. The arrows indicate the direction and extent of sequence obtained from each clone. The construction of the clones was carried out as follows. SAW12 and SAW13 were constructed by isolating the 156bp *Nae*I fragment from pAS20. The fragment was made blunt ended using T4 DNA polymerase and subcloned into M13mp18 cut with *Sma*I. Clones were obtained with the fragment in both orientations using the complementarity test. SAW15 and SAW20 were constructed by isolating the 690bp *Nae*I fragment from pAS20, blunt ending the fragment with T4 DNA polymerase and subcloning the fragment into M13mp18 cut with *Sma*I. Recombinant phage containing the *Nae*I fragment were identified by restriction mapping of RF DNA with *Hin*dIII and *Eco*RI. Clones with the fragment in opposite orientations were identified by the complementarity test. SAW14 was constructed by isolating the 2.4 kb *Hin*dIII-*Xho*I fragment from pAS20. This fragment was subsequently digested with *Hinf*I and the 1.4kb *Hin*dIII-*Hinf*I fragment produced was isolated. This fragment was subsequently made blunt ended using Klenow enzyme and subcloned into M13mp18 cut with *Sma*I. Recombinant phage were identified by restriction mapping of RF DNA and the orientation of the inserts was determined by sequencing.

SAW17 was constructed by isolating the 0.8kb *Pvu*II- *Sma*I fragment from pAS20 and subcloning this fragment into M13mp18 cut with *Sma*I. Clones were obtained with the fragment in both orientations as determined by the complementarity test. SAW18 was constructed by isolating the 400bp *Ban*II fragment from pAS20. The fragment was made blunt ended using T4 DNA polymerase and subcloned into M13mp18 cut with *Sma*I. Recombinant phage were identified by restriction mapping with *Hin*dIII and *Eco*RI and the orientation of the inserts was

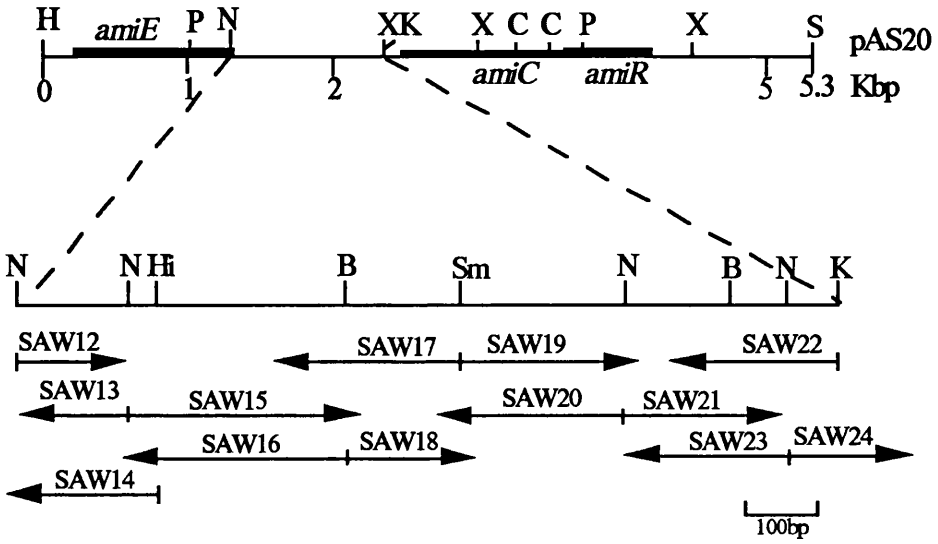


Figure 6.2 The sequencing strategy for the region of DNA between *amiE* and *amiC*. The *NaeI* target shown at the left hand side of the sequencing statergy lies within the last 30bp of the *amiE* coding sequence. Arrows represent the direction and extent of DNA sequence obtained from each clone. Abbreviations for restriction enzyme targets are as follows: H= *Hin*dIII, P= *Pvu*II, X= *Xho*I, K= *Kpn* I, C= *Cla*I, S= *Sal*, B= *Ban* II, N= *Nae* I, Hi = *Hinf*I, Sm = *Sma* I. The *Hinf*I target shown actually represents two very closely spaced *Hinf*I targets.

determined by the complementarity test. SAW16 was constructed in a similar manner to SAW18 except the insert fragment was the 1.5kb *Ban*II fragment from pAS20. SAW19 was constructed by isolating a *Hin*dIII-*Eco*RI fragment from pSW13 which contained the 2.0kb *Sma*I-*Pvu*II fragment from pAS20. This fragment was subcloned directly into M13mp19 cut with *Hin*dIII *and Eco*RI. SAW21 and SAW23 were constructed by isolating the 220bp *Nae*I fragment from pAS20, blunt ending the fragment with T4 DNA polymerase and subcloning the resulting fragment into M13mp18 cut with *Sma*I. SAW22 was constructed by isolating the 500bp *Sma*I-*Kpn*I fragment from pAS20 and subcloning this into M13mp19 cut with *Kpn*I and *Hinc* II. Recombinant phage were identified by restriction mapping with *Hin*dIII and *Eco*RI. SAW24 was constructed by isolating the 1.7kb *Nae*I fragment from pAS20 and subcloning this fragment into M13mp18 cut with *Sma*I. The orientation of the fragment was determined by restriction mapping with *Hin*dIII and *Kpn*I. The DNA sequencing was carried out as described in Chapter 2 using deaza-GTP.

6.4.2 DNA sequence of the region between *amiE* and *amiC* - and sequence analysis

Figure 6.3 shows the DNA sequence obtained using the clones described above and the context of this sequence with respect to the previously sequenced *amiE* and *amiC*. Twenty base pairs downstream of the *amiE* stop codon (1300) is G-C rich axis of dyad symmetry which could represent a transcription termination signal. An open reading frame was identified starting at position 1387 and stopping at position 2502 and a translation of this open reading frame is shown. An analysis of this open reading frame using the third position GC bias plot (Bibb *et al*, 1984) and the codon preference statistic (Gribskov *et al*, 1984) is shown in Figure 6.4. This analysis indicates this is the most likely open reading frame within this region of DNA since there is a large increase in the third position GC bias and the codon preference statistic over the entire ORF and relatively few rare codons are used.

```
                                                          NaeI
                                          amlE ======>    GGC CGG CTG
                                                          Gly Arg Leu

                                                1300                  1320
CCC TAT GAG GGA CTG GAG AAG GAA GCC TGA CGGCAGACGCCGCCAGCCCGG
Pro Tyr Glu Gly Leu Glu Lys Glu Ala ---                    <<<<

                1340                  1360                  1380
CGTGCCGTCGTGCGGCACGCCGTCTCCGCCATTTCCCCCCTGTGGCAGAAGGAGTTTCATCC
<<<<<<<<<<      >>>>>>>>>>>>>>>>

                1400                  1420                  1440
ATG CCT TTT CTG AGC GAC ATG CTC GAC CAG TCC CGC CGG CAG CAG GAC GAG GAA
Met Pro Phe Leu Ser Asp Met Leu Asp Gln Ser Arg Arg Gln Gln Asp Glu Glu

                        1460                  1480
CAG GCC CTG GCG CGG GAG AAT CTT GCC GAG GCA AGT CTG CTC CAG GCC CAC CTG
Gln Ala Leu Ala Arg Glu Asn Leu Ala Glu Ala Ser Leu Leu Gln Ala His Leu

   1500                  1520                  1540
AGT CAC CGC AGC GCC CTG CAC AGC CGT TTC CGT TTC GAC CCG GCG GCG GTG ATG
Ser His Arg Ser Ala Leu His Ser Arg Phe Arg Phe Asp Pro Ala Ala Val Met

            1560                  1580                  1600
GAC TGC CTG CGC GCC GAG GTG CTC GGC CAG GAA CCG GCG CTA CAG GCC GTC GAG
Asp Cys Leu Arg Ala Glu Val Leu Gly Gln Glu Pro Ala Leu Gln Ala Val Glu

                    1620                  1640
GAC ATG CTC AAG GTG GTT CGC GCG GAC ATC GCC GAC CCG CGC CGT CCG CTG TTC
Asp Met Leu Lys Val Val Arg Ala Asp Ile Ala Asp Pro Arg Arg Pro Leu Phe

 1660                  1680                  1700
AGC GCG CTG TTC CTC GGC CCC ACC GGA GTC GGC AAG ACC GAG ATC GTG CGC GCC
Ser Ala Leu Phe Leu Gly Pro Thr Gly Val Gly Lys Thr Glu Ile Val Arg Ala

          1720                  1740                  1760
CTG GCC AGG GCC CTG CAC GGC GAC GCC GAG GGG TTC TGC CGG GTG GAC ATG AAC
Leu Ala Arg Ala Leu His Gly Asp Ala Glu Gly Phe Cys Arg Val Asp Met Asn

                1780                  1800
ACC CTG TCC CAG GAG CAC TAT GCC GCC GCC CTC ACC GGT GCG CCG CCG GGC TAC
Thr Leu Ser Gln Glu His Tyr Ala Ala Ala Leu Thr Gly Ala Pro Pro Gly Tyr

                        1840                  1860
GTC GGG GCG AAG GAG GGC ACC ACC CTG TTG GAG CAG GAC AAG CTG GAC GGC AGT
Val Gly Ala Lys Glu Gly Thr Thr Leu Leu Glu Gln Asp Lys Leu Asp Gly Ser

      1880                  1900                  1920
CCC GGG CGC CCC GGC ATC GTT CTC TTC GAC GAA CTG GAA AAG GCC AGC CCG GAA
Pro Gly Arg Pro Gly Ile Val Leu Phe Asp Glu Leu Glu Lys Ala Ser Pro Glu

                1940                  1960                  1980
GTG GTC CAT GCG TTG CTC AAC GTA CTC GAC AAC GGC CTG CTA CGG GTC GCT TCC
Val Val His Ala Leu Leu Asn Val Leu Asp Asn Gly Leu Leu Arg Val Ala Ser

                        2000                  2020
GGC GAA CGC ACC TAC CAT TTC CGC AAC ACC CTG GTG TTC ATG ACC AGC AAT CTC
Gly Glu Arg Thr Tyr His Phe Arg Asn Thr Leu Val Phe Met Thr Ser Asn Leu

   2040                  2060                  2080
TGC GCC CAT GAG ATC CAG CGC TAC GAC GAG CGT CGC CAG CGC CTG CCC TGG CGC
Cys Ala His Glu Ile Gln Arg Tyr Asp Glu Arg Arg Gln Arg Leu Pro Trp Arg

            2100                  2120                  2140
CTG CTG CCG GTC GGC GGC GAG CGC CGG CGG CGG GAC ATC GAC GGG ATG GTC CGG
Leu Leu Pro Val Gly Gly Glu Arg Arg Arg Arg Asp Ile Asp Gly Met Val Arg

                    2160                  2180
GCC CGG CTG CTG AAG ACC TTC TCG CCG GAG TTC GTC AAT CGT CTC GAT AGC GTG
Ala Arg Leu Leu Lys Thr Phe Ser Pro Glu Phe Val Asn Arg Leu Asp Ser Val

 2200                  2220                  2240
GTC ACC TTC AAC TGG ATC GAA CGC GAC GTC GTC GCG CGC CTG GTC GAG CTG GAG
Val Thr Phe Asn Trp Ile Glu Arg Asp Val Val Ala Arg Leu Val Glu Leu Glu

          2260                  2280                  2300
GTG CAG CGG CTC AAC CGG CGC CTG GAG AAG CAT CGC TGC CGC CTG GAG GCG ACC
Val Gln Arg Leu Asn Arg Arg Leu Glu Lys His Arg Cys Arg Leu Glu Ala Thr

                2320                  2340
CCG GAG GTG CTG GCG AAG ATC GCC CGC GCC GGC TTC GAC CGG CAG TTC GGC GCC
Pro Glu Val Leu Ala Lys Ile Ala Arg Ala Gly Phe Asp Arg Gln Phe Gly Ala
```

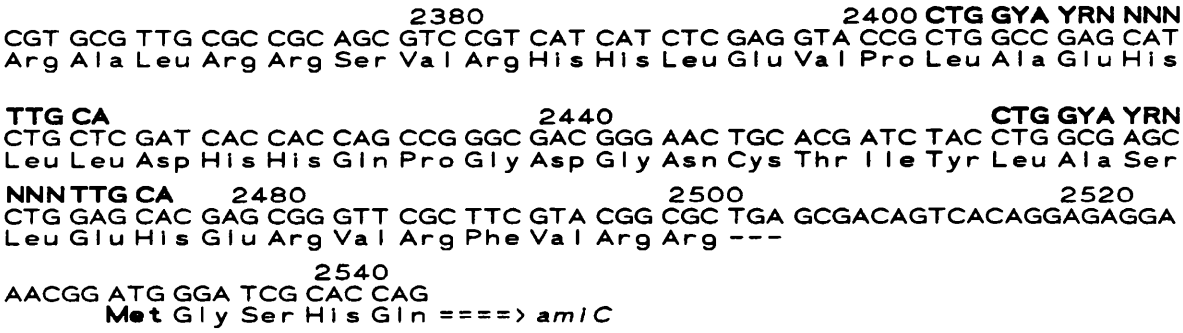


Figure 6.3. The nucleotide sequence of the DNA between *amiE* and *amiC*. The base numbering starts from the unique *Hin*dIII target upstream of *amiE*. Downstream of the *amiE* termination codon is an axis of dyad symmetry between bases 1322 and 1346 which could act as a transcription termination signal. An open reading frame starts at position 1387 and continues to position 2512. The positions of the two *ntrA* dependent promoters are shown with the consensus sequence above at positions 2401-2417 and 2458-2473. A possible ribosome binding site is shown in bold upstream of the open reading frame at positions 1371 to 1378.

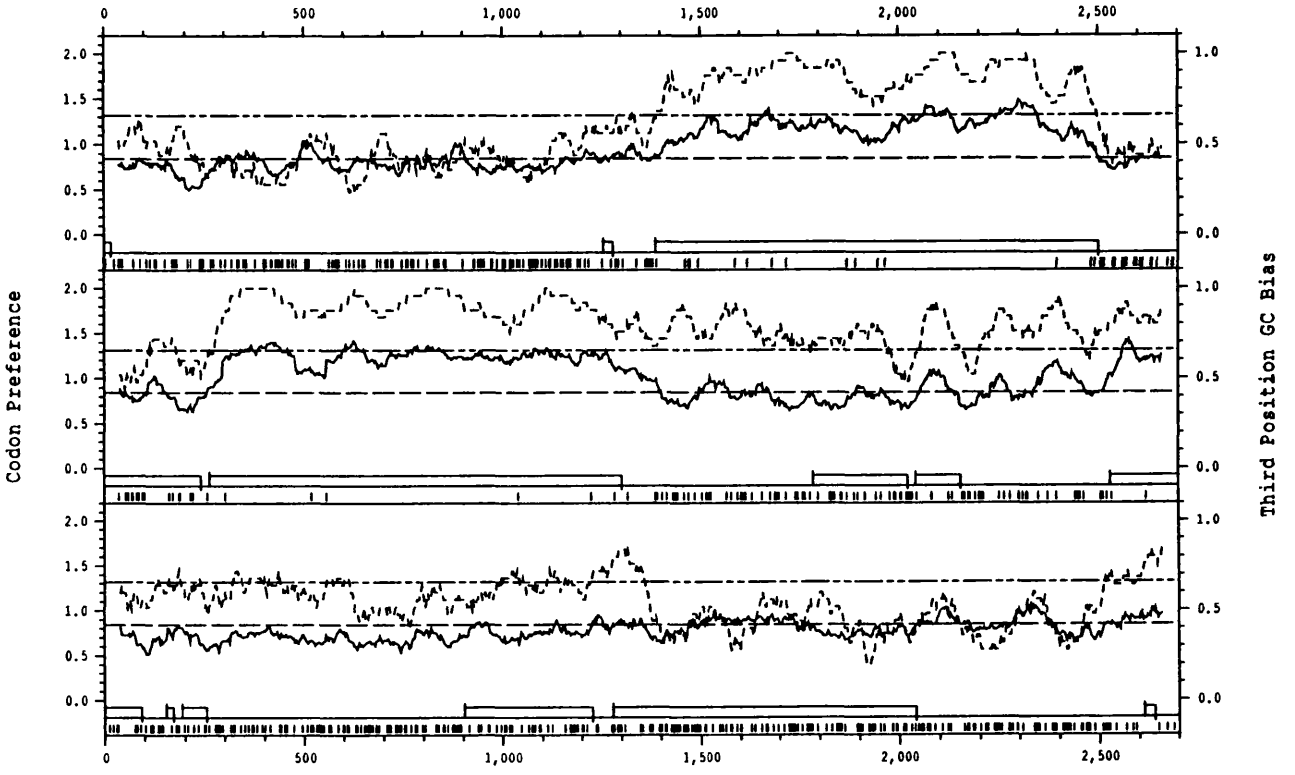


Figure 6.4. Codon preference analysis for the *amiE and amiY* genes. The sequence is numbered from the unique *Hin*dIII target upstream of *amiE*. Each panel represents one reading frame, RF(1) top, RF(2) middle and RF(3) bottom. The continuous line (———) shows the codon preference statistic, the dashed line (-----) shows the third position GC bias and vertical bars below each panel show rare codons. The reference lines at codon preference of 0.84 and GC bias of 0.65 show the values for random sequence with the same base composition. Open reading frames are indicated by boxes at the bottom of each panel. The *amiE* open reading frame is seen in panel two between positions 265 and 1302. The *amiY* open reading frame is seen in panel one between positions 1387 and 2502. The window size and codon usage table used for this analysis were as described in Chapter three.

This analysis has previously been used to predict the correct open reading frames for *amiC* (Chapter 3) and *amiR* (Lowe *et al*, 1989) because of the highly biased codon usage in *P.aeruginosa*. This strongly suggests that this new open reading frame does encode a protein. The open reading frame was provisionally named *amiY* and would code for a 371 amino acid protein of predicted molecular weight 42 kDa. A codon utilisation table for *amiY* is shown in Table 6.8. Upstream of the start codon for *amiY* there is a sequence complementary to the 3' end of the 16s rRNA of *P.aeruginosa* (Toschka *et al*, 1988) which could represent a ribosome binding site (positions 1371-1378).

In the short intergenic region between *amiE* and *amiY* there are no sequences with homology to known consensus promoter sequences. The presence of a possible transcription termination signal upstream of *amiY* could mean this gene is transcribed from the *amiE* promoter and its expression is also regulated by transcription antitermination by *amiR*. However the conserved sequence motifs present in the *amiE* leader sequence and the *bgl* leader sequence (Drew and Lowe, 1989) which define a recognition sequence for transcription antiterminators (*amiR*) are not present upstream of the potential terminator. The two *ntrA* promoter sequences present in the 658bp *Xho*I fragment overlap the C-terminus of the *amiY* ORF and there is a very short intergenic region between *amiY* and *amiC* (25bp)

The only direct evidence that *amiY* exists apart from the sequence analysis is the observation that phage insertions in this region of the PAO1 chromosome render the mutant strains amidase negative (Rehmat and Shapiro, 1983). The attempts to overexpress *amiY* in pSW42 apparently failed and this could be because there was a potential transcription terminator between the *tac* promoter present in the expression vector and *amiY*. Phenotypically the role of *amiY* in amidase regulation would appear to be as a positive controlling element from the phage insertions since its disruption rendered the system amidase negative.

Table 6.8 - Codon utilisation for the *amiY* gene.

Codon	Amino acid	Number	%	Codon	Amino acid	Number	%
TTT	Phe	1	0.3	ATT	Ile	0	0.0
TTC	Phe	14	3.8	ATC	Ile	8	2.2
TTA	Leu	0	0.0	ATA	Ile	0	0.0
TTG	Leu	3	0.8	ATG	Met	7	1.9
TCT	Ser	0	0.0	ACT	Thr	0	0.0
TCC	Ser	3	0.8	ACC	Thr	12	3.2
TCA	Ser	0	0.0	ACA	Thr	0	0.0
TCG	Ser	1	0.3	ACG	Thr	1	0.3
TAT	Tyr	1	0.3	AAT	Asn	3	0.8
TAC	Tyr	4	1.1	AAC	Asn	7	1.9
TAA	Stop	0	0	AAA	Lys	0	0.0
TAG	Stop	0	0	AAG	Lys	8	2.2
TGT	Cys	0	0	AGT	Ser	3	0.8
TGC	Cys	5	1.3	AGC	Ser	9	2.4
TGA	Stop	1	--	AGA	Arg	0	0.0
TGG	Trp	2	0.5	AGG	Arg	1	0.3
CTT	Leu	1	0.3	GTT	Val	3	0.8
CTC	Leu	14	3.8	GTC	Val	13	3.5
CTA	Leu	2	0.5	GTA	Val	3	0.8
CTG	Leu	30	8.1	GTG	Val	10	2.7
CCT	Pro	1	0.3	GCT	Ala	1	0.3
CCC	Pro	4	1.1	GCC	Ala	21	5.7
CCA	Pro	0	0.0	GCA	Ala	1	0.3
CCG	Pro	12	3.2	GCG	Ala	14	3.8
CAT	His	7	1.9	GAT	Asp	2	0.5
CAC	His	8	2.2	GAC	Asp	20	5.4
CAA	Gln	0	0.0	GAA	Glu	7	1.9
CAG	Gln	14	3.8	GAG	Glu	23	6.2
CGT	Arg	7	1.9	GGT	Gly	1	0.3
CGC	Arg	24	6.5	GGC	Gly	15	4.0
CGA	Arg	0	0.0	GGA	Gly	1	0.3
CGG	Arg	14	3.8	GGG	Gly	5	1.3

The percentage values refer to the percentage of the total 371 amino acids used.

Homology searches were carried out with the predicted amino acid sequence of *amiY* and the entire EMBL database in an attempt to ascribe a function to this gene. The analysis showed that the ATP binding subunit of the ATP dependent Clp protease of *E.coli*, encoded by the *clpA* gene (Gottesman *et al*, 1990) had significant homology with *amiY* in some regions. An alignment of the two sequences is shown in Figure 6.5.

The *clpA* gene is a much larger than *amiY* and codes for a protein of 758 amino acids (Gottesman *et al*, 1990), twice the size of AmiY. *clpA* contains two nucleotide binding domains each of which is made up of two separate motifs. The two sequence motifs which form a nucleotide binding domain were first characterised by Walker *et al* (1982) by comparison of a large number of proteins which bind ATP. The consensus sequence has more recently been modified and Gottesman *et al* (1990) have now derived the following consensus for *E.coli* for the first domain: $(\Phi_2\text{-}X_4$)-Gly-X -Gly-Lys-Thr-(Φ-X-X) ; where Φ = isoleucine, leucine, valine, methionine, phenylalanine or tyrosine and X= any amino acid. This domain is known as part A. Part B is generally between 60 and 80 amino acids downstream from part A and has the following consensus $(\Phi)_4$-$(Asp/Glu)_2$. The abbreviations are the same as for part A above.

In the alignment shown in Figure 6.5 the C-terminal ATP binding region of *clpA* is highlighted (PartA + PartB) and this has been aligned with the potential ATP binding Parts A and B of *amiY*. The homology between Part A in both proteins is absolute. The spacing between Part A and Part B is similar in both proteins and there is significant homology in Part B between the proteins and with the consensus. The overall homology between the two proteins is most striking around the potential ATP binding region and tails off at the N-terminus and C-terminus of *amiY*. The amino acid sequence homology between the two proteins between positions 60 and 217, which encompasses the ATP binding motifs is 41% absolute identity and allowing for conservative amino acid changes the homology rises to 58%.

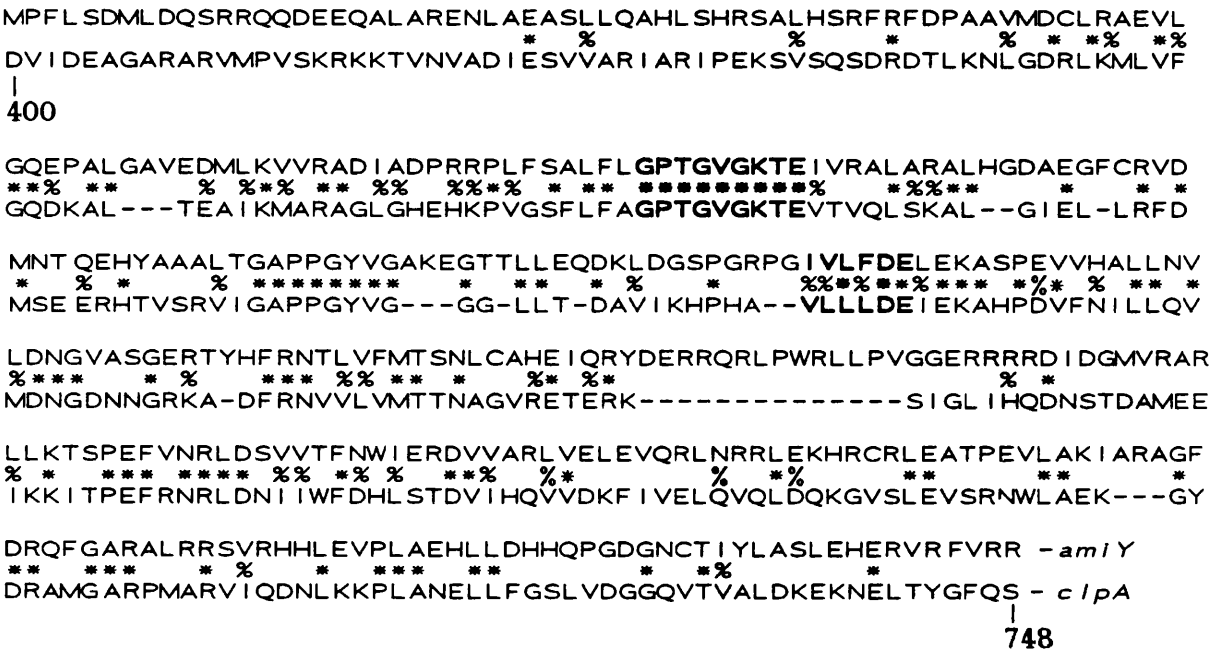


Figure 6.5. Alignment of AmiY with ClpA. Some gaps have been introduced into the ClpA sequence. The sequence in bold indicates two motifs which form the nucleotide binding site. Key: A * indicates complete identity. % indicates conservative amino acid changes. The entire AmiY sequence is shown.

This level of homology strongly suggests that *amiY* has the potential to bind a nucleotide for example ATP via the two conserved motifs described above. The function of *amiY* remains unclear though. Insufficient time was available to construct mutations in *amiY*, and examine the phenotype of *amiY*$^-$ plasmids and it is only possible to speculate on the role of this gene in amidase regulation. *amiY* will be further considered in Chapter seven when constructing a model for amidase regulation.

6.5 Sequencing of the DNA downstream of *amiR* - another ORF?

The DNA sequence of the entire 1.5kb *Xho*I fragment from pJB950 has previously been determined by N.Lowe (personal communication) which includes the published *amiR* sequence. By joining this sequence to the *amiE*, *amiC* and *amiY* sequences this meant that 4.5kb of continuous sequence had now been determined from the original 5.3kb *Hin*dIII-*Sal*I fragment that was originally cloned, containing the amidase genes. The results presented in Chapter 5 with pSW101 and the inducible amidase activity shown with pAS20 when grown under nitrogen limiting conditions, strongly suggested that the 5.3kb fragment encoded all the genes required for inducible amidase expression. It was therefore of some interest to determine the remaining 800bp of DNA sequence downstream of the 1.5kb *Xho*I fragment to determine whether this region of DNA might also encode a protein involved in amidase regulation.

6.5.1 Construction of M13 clones and sequencing strategy

This DNA sequencing was carried out during the final few weeks of experimental work and it was not possible to construct all the clones necessary to sequence both strands of the entire 800bp region. Consequently there are possibly some mistakes in this region of sequence. The sequencing stategy used for this region of DNA is shown in Figure 6.6 and the names of the clones constructed.

The M13 recombinants were constructed as follows: SAW25 and SAW26 were isolated by subcloning the 800bp *Xho*I-*Sal*I fragment from pAS20 into

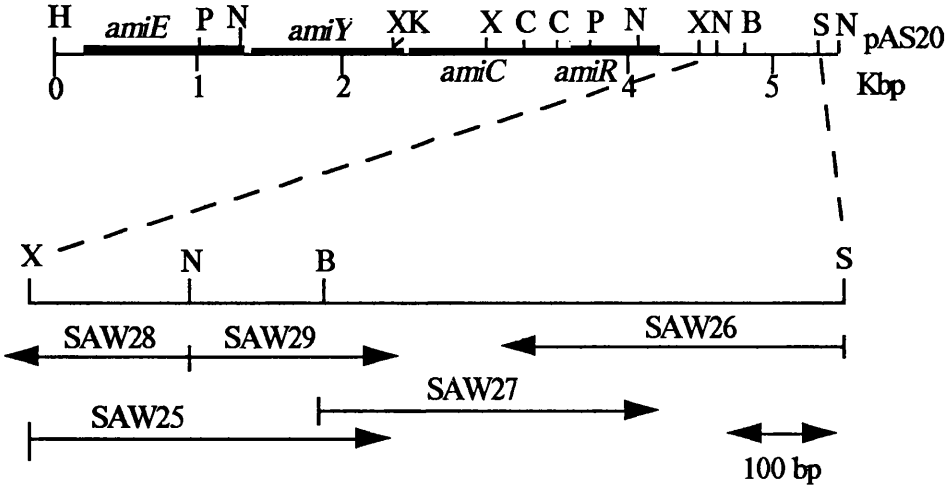


Figure 6.6 The sequencing strategy for the region of DNA between *Xho*I (4560) and *Sal*I (5389) Arrows represent the direction and extent of DNA sequence obtained from each clone. Abbreviations for restriction enzyme targets are as follows: H= *Hin*dIII, P= *Pvu*II, X= *Xho*I, K= *Kpn* I, C= *Cla*I, S= *Sal*I,B= *Bal* I N= *Nae* I.

M13mp18 cut with *SalI*. Recombinant phage were identified by restriction mapping RF DNA with *Hin*dIII and *Eco*RI. The orientation of the inserts was determined by sequencing several recombinants containing inserts. SAW27 was constructed by isolating the 1.4kb *BalI* fragment from pAS20 and subcloning this into M13mp18 cut with *Sma*I. The orientation of the insert in the phage was determined by restriction mapping with *SalI*. SAW28 was constructed by isolating the 530bp *Nae*I fragment from pAS20. This fragment was made blunt ended with T4 DNA polymerase and then subcloned into M13mp18 cut with *Sma*I. Recombinants containing inserts were identified by restriction mapping with *Hin*dIII and *Eco*RI and the orientation of the insert determined by DNA sequencing. SAW29 was constructed by isolating the 870bp *Nae* I fragment from pAS20, blunt ending the fragment with T4 DNA polymerase and subcloning this fragment into M13mp18 cut with *Sma*I. The orientation of the insert was determined by restriction mapping with *SalI*, *Hin*dIII and *EcoR* I.

The DNA sequence obtained for this region of DNA is presented in Figure 6.7 along with the sequence previously determined by N.Lowe (personal communication) for the region 3' of *amiR* down to the *Xho*I target at position 4560. This sequence (Lowe) is derived from the constitutive mutant PAC433 whereas the sequence of the *Xho*I-*SalI* fragment is from PAC1.

6.5.2 Identification of *amiX*

Analysis of the DNA sequence presented in Figure 6.8 was carried out using the third position GC bias statistic and the codon preference plot to try and identify any additional open reading frames 3' of the *amiR* gene. The analysis (shown in Figure 6.8) reveals another open reading frame which has the characteristics of a real gene i.e few rare codons used, high bias of G's and C's in the third position of codons. The open reading frame starts at position 4318 and terminates at position 4833. There is a ribosome binding site upstream of the ORF at positions 4300-4307. A translation of the open reading frame is shown in Figure 6.7. The ORF was provisionally named *amiX* and codes for a 171 amino acid protein with a molecular weight of 18.2 kDa.

```
          4260                        4280                        4300
CTG GGA AAC GAG CCG TCC GCC TGA GCGATCCGGGCCGACCAGAACAATAACAAGAGGGG
Leu Gly Asn Glu Pro Ser Ala ---
          4320                        4340                        4360
TATCGTCATC ATG CTG GGA CTG GTT CTG CTG TAC GTT GGC GCG GTG CTG TTT CTC
           Met Leu Gly Leu Val Leu Leu Tyr Val Gly Ala Val Leu Phe Leu
                    4380                        4400
AAT GCC GTC TGG TTG CTG GGC AAG ATC AGC GGT CGG GAG GTG GCG GTG ATC AAC
Asn Ala Val Trp Leu Leu Gly Lys Ile Ser Gly Arg Glu Val Ala Val Ile Asn
 4420                        4440                        4460
TTC CTG GTC GGC GTG CTG AGC GCC TGC GTC GCG TTC TAC CTG ATC TTT TCC GCA
Phe Leu Val Gly Val Leu Ser Ala Cys Val Ala Phe Tyr Leu Ile Phe Ser Ala
          4480                        4500                        4520
GCA GCC GGG CAG GGC TCG CTG AAG GCC GGA GCG CTG ACC CTG CTA TTC GCT TTT
Ala Ala Gly Gln Gly Ser Leu Lys Ala Gly Ala Leu Thr Leu Leu Phe Ala Phe
                    4540                        4560
ACC TAT CTG TGG GTG GCC GCC AAC CAG TTC CTC GAG GTG GAC GGC AAG GGC CTC
Thr Tyr Leu Trp Val Ala Ala Asn Gln Phe Leu Glu Val Asp Gly Lys Gly Leu
                    4600                        4620
GGC TGG TTC TGC CTG TTC GTC AGC CTC ACC GCC TGC ACC GTG GCG ATC GAG TCG
Gly Trp Phe Cys Leu Phe Val Ser Leu Thr Ala Cys Thr Val Ala Ile Glu Ser
      4640                        4660                        4680
TTC GCC GGC GCC AGT GGT CCG TTC GGC CTG TGG AAC GCG GTC AAC TGG ACA GTC
Phe Ala Gly Ala Ser Gly Pro Phe Gly Leu Trp Asn Ala Val Asn Trp Thr Val
            4700                        4720                        4740
TGG GCG TTG CTC TGG TTC TGT TTC TTC CTG CTG CTG GGG CTG TCC CGC GGC ATC
Trp Ala Leu Leu Trp Phe Cys Phe Phe Leu Leu Leu Gly Leu Ser Arg Gly Ile
                    4760                        4780
CAG AAG CCG GTG GCC TAC CTG ACC CTG GCC AGC GCC ATA TTC ACC GCC TGG TTG
Gln Lys Pro Val Ala Tyr Leu Thr Leu Ala Ser Ala Ile Phe Thr Ala Trp Leu
   4800                        4820                        4840
CCC GGC CTG CTG CTG CTC GGA CAG GTG CTC AAG GCA TAG CAGGAAGTCGGAAAGGG
Pro Gly Leu Leu Leu Leu Gly Gln Val Leu Lys Ala ---
        4860                        4880                        4900
ATGACGGCTTGCCGCCATCCCGTCCCTTCCGAACGCCTAGCCGAGCGGCCAGTTGATCACCA
     4920                        4940                        4960
CGACGGCGTCGTTGTAGTCGTTGTCGGTGCCGTCTTCAGAGCCGACCAGGGCGAAGTTCAGC
   4980                        5000                        5020
TCGTTGGTCAGGATTACCTGTGCCGAGACCAGATCCGAGGGGCGGCCGTTGACGCTGACCTG
5040                        5060                        5080
GACCTGTACCTTGCCACTGCTGCCGGAGTTGAGCACCTGGGTGCCGATGACGGCGTTATTGG
                    5120                        5140                        5160
TGCTTTGCCCGCTGAAGGTCGCGGCCGTGCTCGTTGTTGACCAGCACGTTCACCGTCTGGGT
                    5180                        5200                        5220
TCCGGACGAGTTGGCGAAGGCGGTGACGCCGGAACCTGGTTGTTGGCGGGAAGGGTGAACAC
                  5240                        5260                        5280
TCCTTGTGGTTGCCATGGTGGTATCTCCACTGAATACCTGGCCCCTTCCTTTTCAGGCAGCC
                5300                        5320                        5340
GTCTGGCGCGCGGTATGGCGTGTCGGGAGAAATCCGCAGTCCTTGGCGGCAGGCGATGCGCA
          5360                        5380
GGCAGGAAGGACGCATCGTTCAGCCAATCTACGCCGTCGAC
```

Figure 6.7 Nucleotide sequence of the DNA downstream of *amiR* to the *Sal*I target. The numbering of the bases starts from the *Hin*dIII target upstream of *amiE*. The *amiX* open reading frame runs from positions 4318-4833. The potential ribosome binding site is shown in bold between positions 4300-4307.

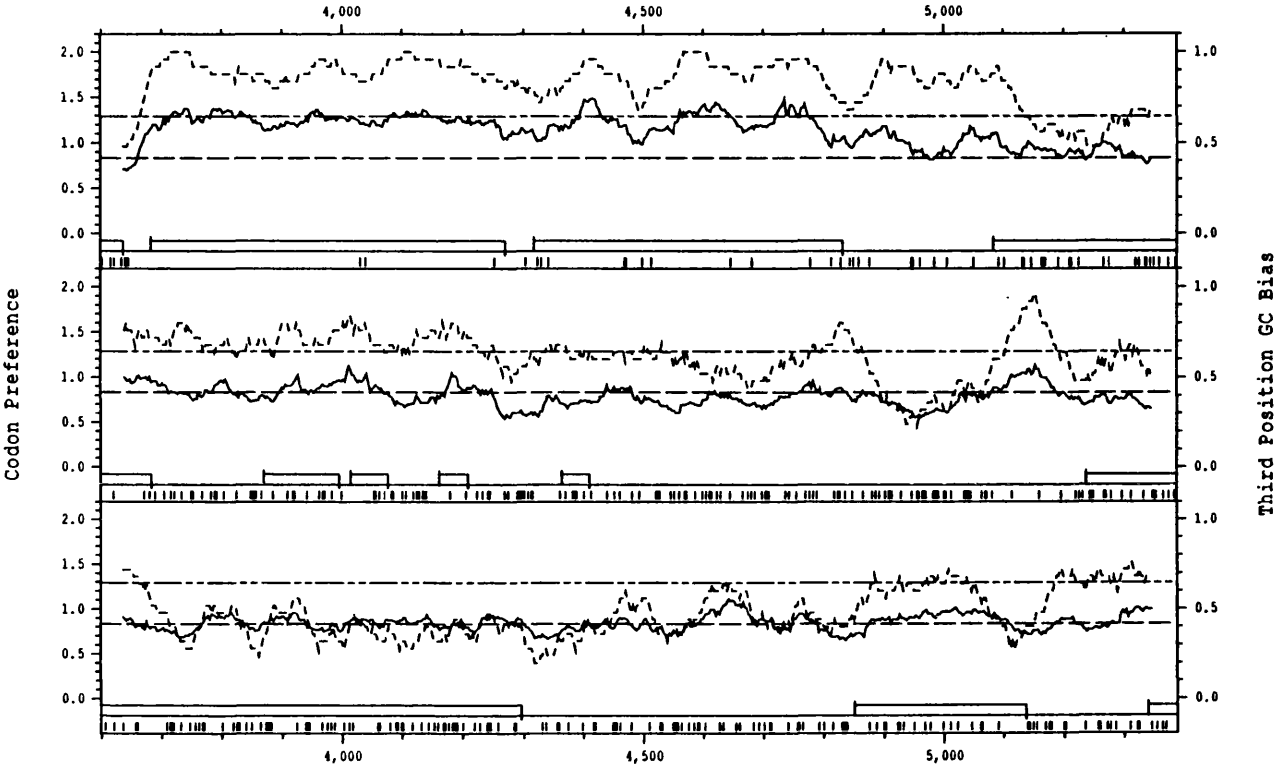


Figure 6.8. Codon preference analysis for the *amiR* and *amiX* genes. The sequence is numbered from the unique *Hin*dIII target upstream of *amiE*. Each panel represents one reading frame, RF(1) top, RF(2) middle and RF(3) bottom. The continuous line (———) shows the codon preference statistic, the dashed line (-----) shows the third position GC bias and vertical bars below each panel show rare codons. The reference lines at codon preference of 0.84 and GC bias of 0.65 show the values for random sequence with the same base composition. Open reading frames are indicated by boxes at the bottom of each panel. The *amiR* open reading frame is seen in panel one between positions 3681 and 4272. The *amiX* open reading frame is also in panel one between positions 4320 and 4830. The window size and codon usage table were as described in Chapter three.

The protein sequence shows no homology with any of the sequences in the EMBL database and as yet there is no known function for *amiX*. A variety of new constructs have been made (e.g pSW3, pSW5) which would have substantial deletions of the *amiX* ORF and these plasmids still express *amiE* in a predictable way. Therefore as far as we can judge, *amiX* does not appear to have an essential role in amidase expression. However, the DNA sequence between the *amiR* and *amiX* open reading frames does not appear to contain any known promoter sequences suggesting the *amiX* ORF might well be coordinately transcribed with *amiC* and *amiR*.

6.6 Analysis of the Transcription Patterns of the Amidase Genes by Northern Analysis

An investigation of the transcription patterns of the amidase genes was essential if a model for the induction process was to be constructed. It was hoped that Northern analysis would indicate the following: (i) the size of the *amiE* transcript; ii) whether *amiC*, *amiR*, and *amiX* were transcribed as part of a polycistronic operon and the size of this transcript; iii) how *amiY* was transcribed.

RNA was isolated from *E.coli* strains JA221 and ET8045 (*ntrA*$^-$) containing plasmid pJB950 grown under nitrogen excess and nitrogen limiting conditions, from PAC1 grown under non-inducing and inducing conditions and from pSW101 in strain PAC452 grown under similar conditions. The RNA (10μg per lane) was run on two separate 1% formaldehyde agarose gels along with BRL RNA molecular weight marker ladders and the gels blotted onto nylon membranes as described in Chapter 2. The first blot was probed with the 580bp *Pst*I-*Pvu*II fragment within the *amiE* gene and the second blot was probed with the 1.5kb *Xho*I fragment which overlaps the *amiC*, *amiR* and *amiX* open reading frames. All the Northern blots described in this Chapter were hybridised at extremely high stringency (50°C, 50% formamide) because of the high GC content of *P.aeruginosa* DNA.

The results from the Northern blot hybridised with the *amiE* probe are shown in Figure 6.9. Part A shows a 2 hour exposure of the autoradiograph and part B shows an overnight exposure. Both are included since the levels of the transcripts present in the different samples varies greatly. Lane 1 (parts A + B) contains RNA isolated from JA221 pJB950 grown in excess nitrogen. A major transcript of 1.3kb is seen in part B and there is a minor amount of hybridisation close to the 16s and 23s rRNA bands.

A consensus *E.coli* promoter sequence has previously been identified upstream of the *amiE* gene (Drew and Lowe, 1989) which probably identifies the 5' end of the *amiE* transcript and a potential transcription terminator structure has been identified downstream of the *amiE* gene (see Figure 6.3). With these transcription starts and stops the *amiE* transcript would be expected to be 1.3kbp long. Therefore the major transcript seen at 1.3kb in lane 1 corresponds to the *amiE* transcript. Lane 2 contains RNA from JA221 pJB950 grown under nitrogen limiting conditions. There is a much larger amount of the transcript at 1.3kb in part A. With the longer exposure of this lane shown in part B there also appears to be a band just below the 23s rRNA band and a further very faint band at approximately 5.0kb. These larger transcripts could correspond to transcription reading through the terminator downstream of the *amiE* gene. It would appear that these larger transcripts are relatively unstable since there appears to be substantial degradation of the RNA in part B.

Lanes 3 and 4 contain RNA isolated from ET8045 pJB950 grown in the presence and absence (limiting) of nitrogen respectively. They both show the 1.3kb transcript in part A and the presence of the two larger bands described for lane 2 in partB. This result confirms that the removal of *ntrA* from *E.coli* increases the amount of *amiE* transcription and amidase activity. Lane 5 and lane 6 contain RNA isolated from PAC1 grown in lactate in the absence and presence of lactamide as inducer respectively.

The non-induced PAC1 RNA (lane 5) shows some non-specific hybridisation to the 16s and 23s rRNA bands and no evidence of the *amiE* transcript. The

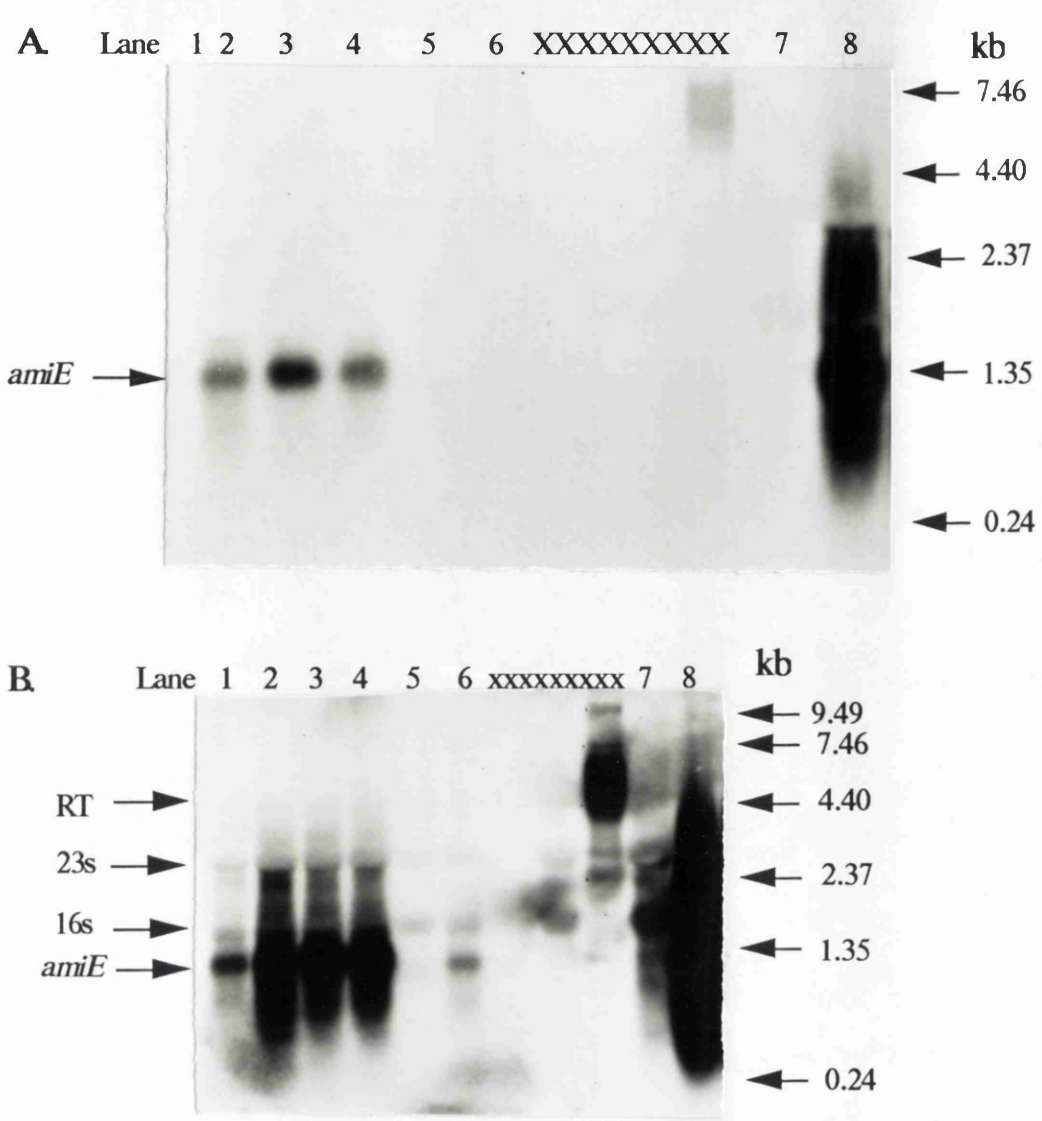


Figure 6.9. Northern analysis using the *Pst* I- *Pvu* II *amiE* probe. Part A shows a 2 hour exposure and part B shows an overnight exposure of the same filter. The photographs are not printed to the same magnification. RNA samples were loaded as follows: Lane 1, JA221 pJB950 + nitrogen; lane 2, JA221 pJB950 - nitrogen; lane 3, ET8045 pJB950 + nitrogen; lane 4, ET8045 pJB950 - nitrogen; lane 5, PAC1 non-induced (lactate); lane 6, PAC1 induced (lactate/lactamide); lane 7, PAC452 pSW101 non-induced; lane 8, PAC452 pSW101 induced. Molecular size markers are shown down the left hand side of each picture. RT stands for read-through transcripts having an approximate molecular weight of 5.0kb, 23s indicates the position of the 23s rRNA band, 16s indicates the position of the 16s rRNA band and *amiE* indicates the 1.3kb *amiE* transcript.

induced PAC1 RNA (lane 6) shows hybridisation to the rRNA bands but also shows the 1.3kb *amiE* transcript however this band is very faint compared with the other lanes. This result is expected since under these inducing growth conditions, PAC1 only produces 2.3 units of amidase activity, which is considerably lower than the activity seen with the other strains used to prepare RNA. The induced PAC1 RNA does not show any evidence of read through transcription producing larger transcripts, however the larger transcripts are only minor species in lanes 2,3 and 4, and so are probably beyond the detection limits with the PAC1 RNA.

Lane 7 and lane 8 contain RNA isolated from PAC452 pSW101 grown in lactate in the absence and presence of lactamide inducer respectively. This strain produces up to 50 units of amidase activity under inducing conditions and would be expected to show large amounts of the *amiE* transcript. Even under non-inducing conditions this strain produces up to one unit of amidase activity. With the non-induced RNA, the 2 hour exposure (part A) shows no evidence of the *amiE* transcript although there is some hybridisation to the rRNA bands. The overnight exposure shows a faint transcript at 1.3kb, a strong band corresponding to the 16s rRNA and a faint band just below the 23s rRNA. Lane 8 contains the induced RNA and shows an unexpected result. The 2 hour exposure shows a major transcript at 1.3kb corresponding to the *amiE* transcript but also a smear which hybridises with the probe which starts at approximately 5.0kb. This smear is seen more clearly in the overnight exposure. The smear is discontinuous (Part A) and is interrupted at the points where the 23s and 16s rRNAs would appear on the membrane. This suggests that the presence of the ribosomal RNAs (which are the major RNA species) is either excluding the hybridising RNA from this region of the gel during electrophoresis or is possibly saturating the membrane in this region reducing the transfer of the hybridising RNA forming the smear.

Transcripts bigger than 1.3kb could arise in two possible ways. Firstly transcription of *amiE* might be originating from a promoter upstream of the consensus *E.coli* promoter. This possibility can be excluded since in section 6.7 the 5' end of the *amiE* transcript is mapped to the *E.coli* like promoter. Secondly,

transcription could be proceeding beyond the potential terminator downstream of *amiE* and reading through *amiY, amiC, amiR* and possibly *amiX.* A transcript reading through all these genes would have a size of at least 4.7kb which agrees closely with the largest transcripts hybridising with the probe in lane 8 which have a calculated size of 5.0kb. This would indicate there is a transcription termination site approximately 300bp downstream of *amiX.* Since the RNA in lane 8 hybridised as a smear this would suggest that large transcripts produced by read-through of the terminator downstream of *amiE* are unstable and are degraded to produce a continuous smear which extends down to RNA sizes of as little as 230 bases. The bands seen just below the 23s rRNA in lanes 2,3 and 4 could represent read through transcripts which have been degraded to a discrete length, which would suggest processing of the RNA. Alternatively the 23s rRNA species could be affecting the migration of the degraded read through transcripts, such that there is a clustering of degraded transcripts just in front of the 23s rRNA band.

These results indicate that transcription originating from the *amiE* promoter has more than one possible termination site. As well as the termination sequence in the *amiE* leader region, there appears to be a major termination site immediately downstream of the *amiE* gene, possibly at the axis of dyad symmetry identified in Figure 6.3. In addition, there is also at least one other transcription termination site downstream of *amiX.* It would thus appear that a minority of *amiE* transcripts extend almost the complete length of the cloned DNA fragment.Resulting in a polycistronic transcript including all the amidase genes. To investigate these conclusions further a second Northern blot and hybridisation was carried out using the 1.5kb *Xho*I fragment as a probe and the results are shown in Figure 6.10.

The RNA samples used for this blot were identical to those used for the first blot probed with *amiE.* What is most striking about this second blot is that it looks very similar to the first blot, probed with an *amiE* specific DNA fragment, except that the 1.3kb *amiE* transcript is not seen.

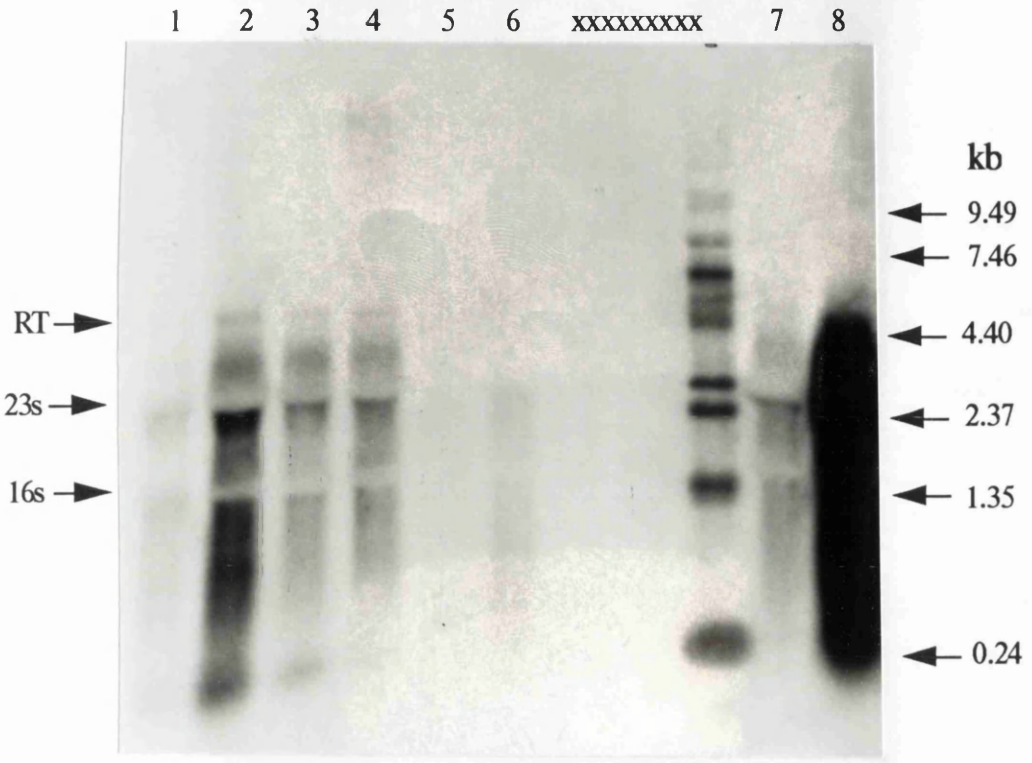


Figure 6.10. Northern analysis using the 1.5kb *Xho*I *amiCR* probe. RNA samples were loaded as follows: Lane 1, JA221 pJB950 + nitrogen; lane 2, JA221 pJB950 - nitrogen; lane3, ET8045 pJB950 + nitrogen; lane 4, ET8045 pJB950 - nitrogen; lane 5, PAC1 non-induced (lactate); lane 6, PAC1 induced (lactate/lactamide); lane 7, PAC452pSW101 non-induced; lane 8, PAC452 pSW101 induced. Molecular size markers are shown down the left hand side of each picture. RT stands for read-through transcripts having an approximate molecular weight of 5.0kb, 23s indicates the position of the 23s rRNA band, 16s indicates the position of the 16s rRNA band.

The first lane contains JA221 pJB950 grown under nitrogen excess conditions and shows a smear beginning at approximately 5.0kb that goes down to 230 bases. Once again there are apparent gaps in the smear corresponding to the location of the rRNA bands and just underneath the 23s rRNA band is an apparent band which could correspond to a discrete transcript. The 5.0kb transcript presumably corresponds to a mRNA encoding *amiE*, *amiY*, *amiC*, *amiR*, and *amiX* since an identical sized transcript is seen when the *amiE* probe is used. A similar result is seen in lane 2 with JA221 pJB950 grown under nitrogen limiting conditions except there is considerably more of all the transcripts. This shows that transcription of *amiC* and *amiR* is increased under nitrogen limiting conditions. Presumably this would lead to increased *amiE* transcription and therefore more read through transcription hence the increase in the amount of the 5.0kb transcript, corresponding to read through of the whole operon.

The results from ET8045 (*ntrA*$^-$) pJB950 (lanes 3 and 4) are similar to the JA221 pJB950 (-N) result. A smear running from 5.0kb to 200 bases is visible. This result strongly suggests that *ntrA* plays a role in down regulating amidase expression and this repression appears to effect the read through transcription from the *amiE* promoter. Lane 5 contains RNA isolated from PAC1 grown under non-inducing conditions and shows no evidence of transcripts hybridising to the 1.5kb *Xho*I fragment probe. Lane 6 contains RNA isolated from PAC1 grown under inducing conditions and shows a very faint smear similar to the smears seen in lanes 1-4. Lane 7 contains RNA isolated from PAC452 pSW101 grown in lactate and shows a similar hybridisation pattern to the first four lanes with a faint band at approximately 5.0kb and a smear of degraded RNA. Once again there is an apparent band below the position of the 23s RNA and there are gaps in the smear at the positions of the 23s and 16s rRNA's. Lane 8 contains RNA isolated from PAC452 pSW101 grown under inducing conditions and shows an intense smear which starts at approximately 5.0kb and continues to sizes less than 230 bases.

From its size , this smear presumably corresponds to transcripts which originate from the *amiE* promoter, read through the potential terminator at the 3' end of *amiE* and continue through all the amidase genes. A similar hybridisation

pattern is seen with the *amiE* probe (see Figure 6.9) except that the smaller 1.3kb *amiE* transcript is also seen. Since a discrete band corresponding to the full length transcript for all the amidase genes it not clearly seen, this suggests that either this large transcript is particularly unstable and is turned over rapidly in the cell or the RNA sample(s) were generally degraded upon isolation. The second alternative seems unlikely since examination of the formaldehyde gels following electrophoresis indicated that the rRNA bands were intact and undegraded and secondly the 1.3kb *amiE* transcript appears as a discrete undegraded band on the autoradiographs.

There are three possible explanations for the appearance of an apparent band below the 23s rRNA when probing with either the *amiE* probe or the 1.5kb *Xho*I fragment. Firstly, full length transcripts could be processed giving rise to a smaller transcript. Secondly, it has been shown that the *ntrA*-dependent promoter elements can down regulate amidase expression, and this could be caused by preventing transcription beyond these promoter elements. Thus, these promoters could represent a major site of transcription termination. Since these promoters are approximately 2.3kb downstream from the *amiE* promoter, termination at these promoters would produce a 2.3kb transcript of a similar size to the apparent band seen below the 23s rRNA. This is an attractive explanation for the appearance of the 2.3kb band but unfortunately the 1.5kb *Xho*I fragment also hybridises with this band and the probe fragment lies approximately 650bp downstream of the proposed termination site. Similarly the 2.3kb band can not represent transcripts originating from the *ntrA* dependent promoters since the *amiE* probe also hybridises with the band. The third alternative is that the band represents a migration anomaly or some phenomenon occurring during the blotting process and actually corresponds to degraded forms of the full length transcript encoding all the amidase genes.

If the smear and the band below the 23s rRNA was a mixture of randomly degraded mRNA's which encoded all the amidase genes then two probes separated by a large distance within the amidase operon should both produce a similar

smear pattern with a band below the 23s rRNA. Secondly, if the band below the 23s rRNA represented a processed form of the full length mRNA then with two probes separated by a distance greater than the 2.3kb band, only one probe should hybridise with it.

The two probes used so far (*Pst* I-*Pvu*II and the 1.5kb *Xho*I fragment), which both hybridise with this band are separated by a distance of 1.7kb which is less than the size of the band (See Figure 6.11). For these two probes to overlap a processed transcript, originating from the *amiE* promoter, would require there to be a processing site within the 3' end of the *amiE* transcript but 5' of the *Pvu*II (1050) target and a second site within the 1.5kb *Xho*I fragment. This seems highly unlikely, but to confirm that this was not the case a Northern blot containing non-induced and induced RNA (20μg) isolated from PAC452 pSW101, was probed with a *NaeI* fragment which lies 3' of the *amiR* gene. The location of this probe with respect to the *amiE* probe is shown in Figure 6.11 along with the results of the Northern blot.

Lane 1 contains non-induced RNA isolated from PAC452 pSW101 and shows a single band with a calculated size of 500 bases. This transcript is produced irrespective of amide inducer and could represent transcription from a separate promoter within the *Xho*I-*Sal*I fragment which is terminating within pKT231 vector sequences. Lane 2 contains induced RNA isolated from PAC452 pSW101 and shows a smear between 5.0kb and 240 bases. As previously, the 23s and 16s rRNA have quenched the signal in these regions. Using the 3' *Nae*I probe the most intense signal is seen between 2.6 and 1.3 kb.

Since all the probes used hybridise with the induced RNA to give a characteristic smear this confirms that the smear corresponds to degraded forms of the full length transcript which encodes all the amidase genes. The bands below 23s rRNA (and 16s rRNA to a small extent) cannot represent processed forms of the full length transcript since both the *Pst*I-*Pvu*II probe and the *Nae*I probe light up these bands and the probes are physically separated by a distance greater than

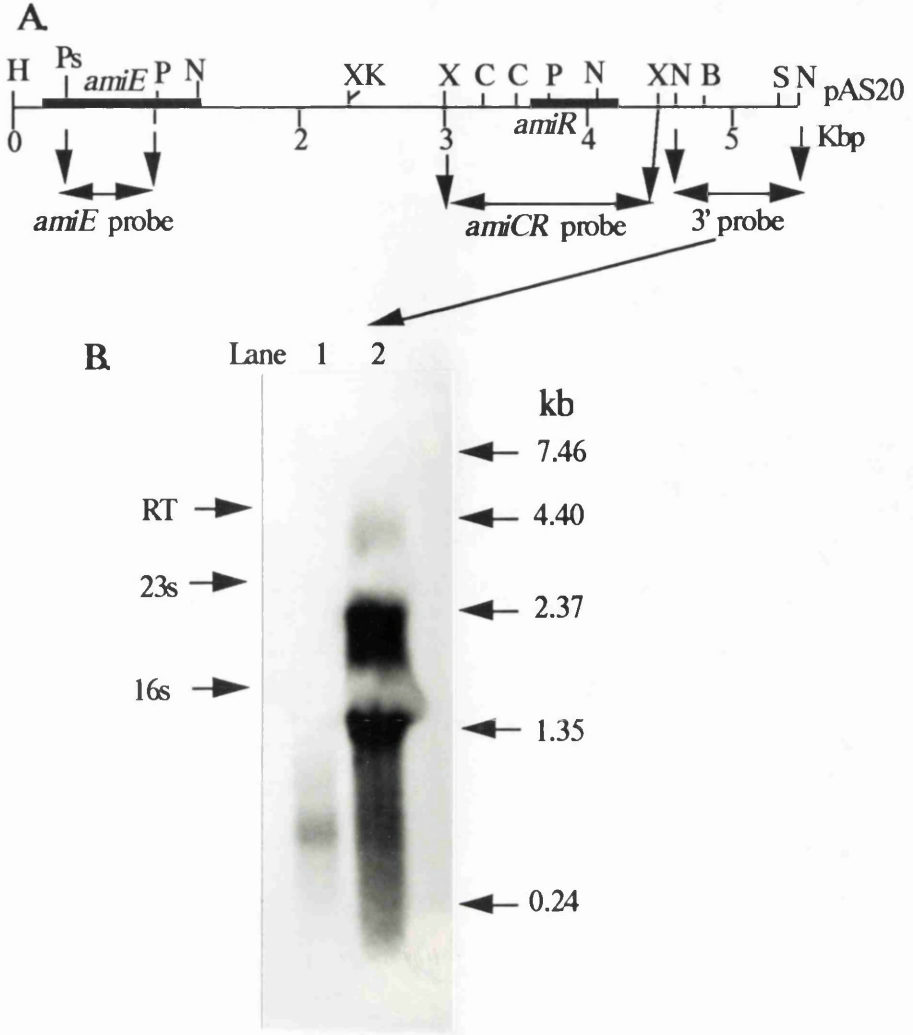


Figure 6.11. Northern analysis using the 3' *Nae*I probe. Part A: Location of the *Nae*I probe, the *amiE* probe and the *amiCR* probe. Part B: Northern analysis using the 3' *NaeI* probe. RNA samples were loaded as follows: Lane 1, PAC452pSW101 non-induced; lane 8, PAC452 pSW101 induced. Molecular size markers are shown down the right hand side of the picture. RT stands for read-through transcripts having an approximate molecular weight of 5.0kb, 23s indicates the position of the 23s rRNA band, 16s indicates the position of the 16s rRNA band.

the size of either of the two bands. The possibility that the 2.3kb band represents transcripts terminating at the ntrA dependent promoters when using an *amiE* probe, and transcripts originating from the ntrA promoter region when using the 1.5kb *Xho*I fragment probe can be excluded since the band is seen in an *ntrA*$^-$ host where the termination event should not occur.

In bacteria mRNA is generally degraded from the 5' end and therefore one would expect that a probe which hybridised to the 5' end of the mRNA would only hybridise to full length or partially degraded mRNA's. However, this does not seem to be the case with the amidase operon transcript since a 5' probe lights up a degraded smear in a manner similar to a 3' probe. The reason for this is not clear. However it is noticeable that with a 5' probe the intensity of the smear in the high molecular weight range is more intense than with the 3' probe (*cf.* Figure 6.9 lane 8 and Figure 6.11 lane 2), suggesting an overall trend for degradation in a 5' to 3' direction.

In conclusion, it would appear that transcription proceeding from the *amiE* promoter, under inducing conditions, has several fates. Firstly it can terminate immediately after the *amiE* gene producing a mRNA transcript of 1.3kb which appears to be relatively stable. Secondly transcription can proceed through the terminator downstream of *amiE* and continue through *amiY*, *amiC*, *amiR* and *amiX*, producing a transcript of approximately 5.0kb which must terminate shortly after the 3' end of *amiX*. This full length transcript appears to be less stable than the *amiE* transcript which could represent a specific control mechanism to ensure regulator gene expression is switched off rapidly. The two *ntrA* promoters do not appear to promote transcription to any significant extent since no discrete smaller transcript appears to be produced which is specific for an *ntrA*$^+$ host under nitrogen limiting conditions. In fact the main role of these promoters would appear to be as a negative control system in which the unactivated NtrA-RNA polymerase complex bound to these promoter(s) reduces transcription of *amiC* and *amiR* from the *amiE* promoter. Removal of NtrA or activation of closed to open complex by NtrC under nitrogen limiting conditions appears to relieve this repression and

cause the appearance of more full length transcripts reading through *amiC*, *amiR* and *amiX*.

Since the *ntrA* dependent promoters could repress transcription originating from the *amiE* promoter one would expect to detect transcripts of approximately 2.3kb, starting from the *amiE* promoter and terminating at the *ntrA* dependent promoters. No such transcripts have been detected using the *amiE* probe. However this could be because such transcripts are being masked on the autoradiographs by the clustering of degraded full length transcripts below the 23s rRNA. Cousens *et al* (1987) showed that there is promoter activity within the 658bp *Xho*I fragment, albeit weak, and it could be the case that the *ntrA* dependent promoters are bifunctional and have some residual promoter activity even under nitrogen excess growth conditions.

Alternatively there could be another type of promoter in this region which does not match any obvious consensus sequence. A low level of transcription from a promoter immediately upstream of *amiC* and *amiR* might ensure AmiC and AmiR are produced in small quantities in readiness for amide inducer to which the two proteins would then respond. Unfortunately low level transcription from this region of DNA has not been detected by Northern analysis and this could be for the same reason that transcripts terminating at the ntrA dependent promoters were not detected. Transcripts starting within the 658bp *Xho*I fragment and continuing to the unidentified termination site 3' of amiX would have an approximate size of 2.3kb, the same size as the cluster of degraded full length transcripts, which would mask the small number of transcripts originating from the promoter element within the 658bp *Xho*I fragment.

In the next section the mapping of the *amiE* promoter is described and this is followed by mapping of the 3' end of the *amiE* transcript which confirms that read through transcription occurs.

6.7 Mapping of the 5' end of the *amiE* transcript

Drew and Lowe (1989) had previously identified a consensus *E.coli* promoter upstream of *amiE*, by sequence homology. They proposed that transcription from

this promoter was constitutive and in the absence of AmiR terminated at the rho-independent transcription terminator between the promoter and *amiE*. To confirm that this promoter was used in *E.coli* and *P.aeruginosa* and to identify the start of transcription primer extension analysis (Sambrook *et al*, 1989) was carried out.

An oligonucleotide (oligoE) was synthesised with a sequence complementary to bases 180-199 shown in Figure 6.13. The oligonucleotide was ^{32}P end-labelled and used in primer extension analysis as described in Chapter two. To provide an accurate size marker and also to identify the base corresponding to the transcription start point, oligoE was used in a sequencing reaction with a complementary M13 clone encompassing the entire leader region. In this manner the exact base corresponding to the transcription start point is identified by examining the sequence ladder at the point of the extension product.

Primer extensions were carried out with RNA isolated from PAC1 (non-induced and induced), JA221 pJB950 and PAC452 pSW101 (induced) and the results are shown in Figure 6.12. Lanes 1-4 contain the sequencing ladder loaded in the order TGCA. Lane 5 contains RNA isolated from PAC1 non-induced and shows a primer extension product of 72bp which corresponds to the A of the sequence CAT at position 128 in Figure 6.13. This primer extension product lies at the appropriate position downstream of the *E.coli*-like promoter showing that this promoter is used in *P.aeruginosa*. The presence of the extension product in a non-induced culture

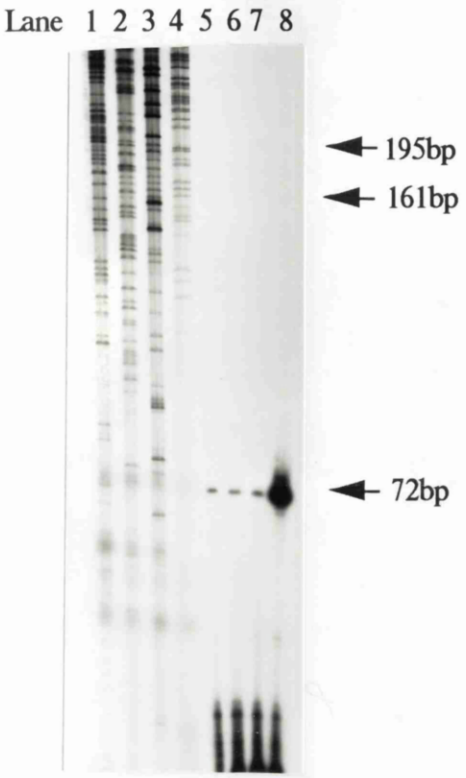


Figure 6.12. Primer extension analysis on the *amiE* promoter region. Primer extension reactions and sequencing reactions used oligoE (see Figure 6.13). Samples were loaded as follows : Lanes 1-4, sequencing reactions loaded in the order T G C A; RNA samples were as follows: lane 5, PAC1 non-induced; lane 6, PAC1 induced; lane 7, JA221 pJB950; lane 8, PAC452 pSW101 induced. The major extension product in all samples is 72bp which maps to the A of the sequence CAT downstream of the consensus *E.coli* promoter (see Figure 6.13). In lanes 7 and 8, two minor extension products are seen with sizes of 195bp and 161bp.

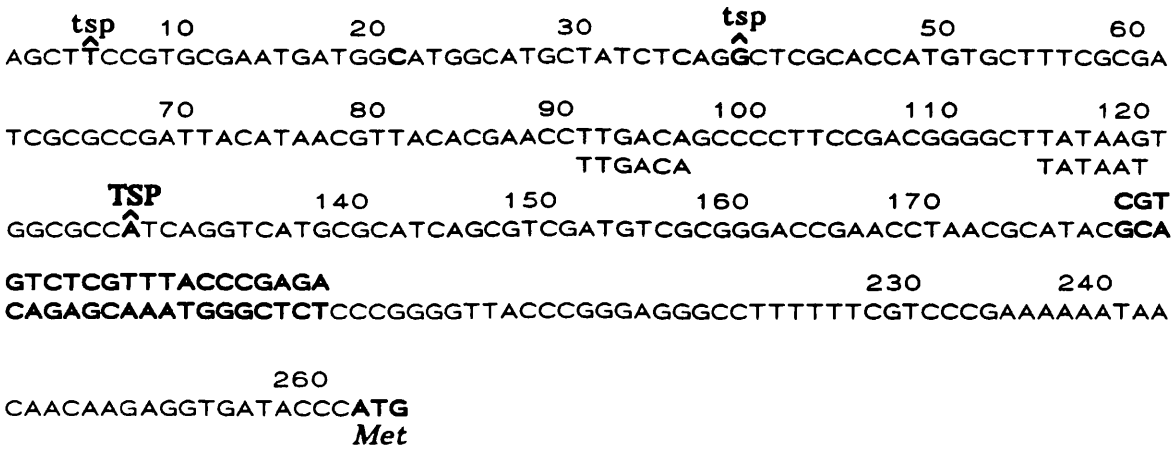


Figure 6.13. Location of oligoE and the primer extension products obtained using this oligonucleotide. The sequence shown runs from the unique *Hin*dIII target upstream of *amiE* to the *amiE* start codon. The sequence of oligoE is shown in bold above the sequence it hybridises to. The position of the *E.coli*-like promoter is shown with the consensus sequence underneath. TSP indicates a transcription start point. The major transcription start point is shown at position 128. Two minor transcription start points were also identified at position 5 and 38.

confirms that transcription from the promoter is constitutive. Lane 6 contains induced RNA isolated from PAC1 and produces an identical extension product of equal intensity, confirming that the induction process is regulated by transcription antitermination and does not involve increased transcription from the *amiE* promoter. Lane 7 contains RNA isolated from JA221 pJB950 and also produces a major extension product of 72 bp. There is also an extension product of 161bp which maps to base 38 shown in Figure 6.13. This is a minor extension product which represents approximately 5% of the total extension products. Lane 8 contains RNA isolated from PAC452 pSW101 induced and produces a major extension product at 72 bp. A small ladder of extension products is seen below the major band which could indicate some degradation of the RNA or premature termination of the reverse transcriptase in the extension reaction. A minor extension product with a length of 195bp is seen which maps to base 5 in Figure 6.12. This extension product might arise from a weak vector promoter adjacent to the *Hin*dIII site.

The most striking observation from this primer extension is the difference in amounts of extension products seen with JA221 pJB950 and PAC452 pSW101. It has previously been assumed that amidase expression from recombinant plasmids in *P.aeruginosa* is much higher than in *E.coli* because of poor expression of *amiR*. Yet in this assay the expression from the *amiE* promoter is AmiR independent and constitutive. pJB950 in JA221 would be expected to have a copy number of approximately 40-50 (H.Richards, personal communication) at the time the RNA was isolated, whereas pSW101 only has a copy number of 13 (Frey and Bagdasarian, 1989), and yet the primer extension signal from PAC452 pSW101 RNA is approximately ten times higher than that with pJB950 in *E.coli* JA221. This observation is even more surprising when one considers that the promoter involved is almost a consensus *E.coli* promoter.

This effect could be due to two reasons: firstly, the *amiE* promoter has a single mismatch with the consensus *E.coli* promoter in the -10 region which could lead to poor recognition in *E.coli*. Secondly, there could be some form of additional control by a CAP like protein in *P.aeruginosa* since it is known that amidase in

P.aeruginosa is subject to catabolite repression and the PAC452 pSW101 culture was catabolite derepressed. Previous studies using cloned amidase genes in *E.coli* have shown no effect of the enteric catabolite repression system on amidase expression (Cousens *et al,* 1987; Drew and Lowe, 1989). Taking into account the differences in copy numbers of the two plasmids, there is probably up to a fifty fold increase in the transcription from this promoter in *P.aeruginosa* compared to *E.coli.* It would have been of interest to isolate RNA from a catabolite repressed culture of PAC452 pSW101 and perform primer extension on the *amiE* promoter to see whether the increased transcription in *P.aeruginosa* was due to a positive control effect involving catabolite repression, however time did not allow this experiment out be carried out.

6.8 Mapping of the 3' end of the *amiE* transcript

The results from the Northern analysis described in section 6.6 indicated that transcription from the *amiE* promoter terminated either shortly after the *amiE* gene or alternatively read through this termination site and proceeded through the rest of the amidase genes. The sequence downstream of the *amiE* gene revealed an axis of dyad symmetry which could represent a transcription terminator. No potential promoter elements had been identified between *amiE* and the following gene, *amiY* and so an experiment was designed to map the 3' end of the *amiE* transcripts and show whether read through transcription was occurring at this potential terminator. In addition the presence of any *amiY* promoter sequences would be detected.

This experiment was carried out using S1 nuclease mapping (Burke, 1984) and used three different probes. The design of the experiment is illustrated in Figure 6.14. Initially, two probes were used which differed in size and both overlapped the potential terminator region at the 3' end of the *amiE* gene. The 5' end of each probe started from the same point within the *amiE* gene but the first probe was only 482bp long and the second probe was 746bp long. Since the probes were continuously labelled they had the potential to map the 3' end of the *amiE* transcript but they also had the potential to map the 5' end of any transcript

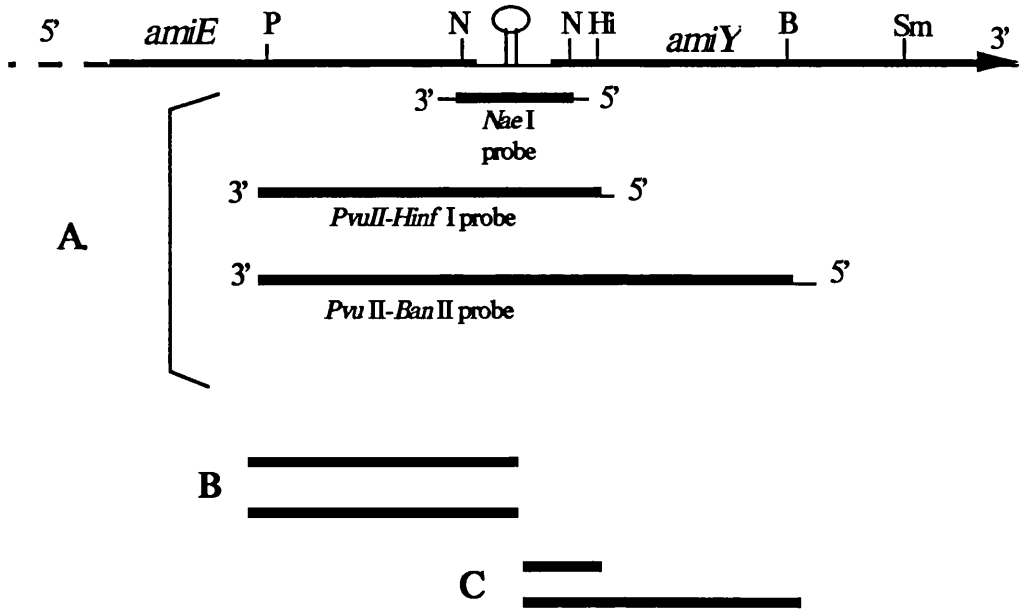


Figure 6.14. Experimental design for mapping the 3' end of the *amiE* transcript and to also detemine whether there was a seperate promoter for *amiY*. Part A shows the three probes used. The *NaeI* probe was used for fine mapping of the 3' end of the *amiE* transcript. The *Pvu* II-*Hinf* I probe and the *Pvu* II-*Ban*II probe were used for low resolution mapping. Part B indicates the type of S1 products expected with the two large probes when mapping the 3' end of a transcript. Part C indicates the type of S1 products that would be expected when mapping the 5' end of a transcript. The fine lines at the end of the probes signify the M13 derived sequences in the probes. The stem loop structure 3' of *amiE* indicates the axis of dyad symmetry previously identified (see Figure 6.3). Abbreviations for restriction sites are as follows: P= *Pvu* II, N= *Nae* I, Hi = *Hinf* I, B= *Ban* II and Sm= *Sma* I.

starting immediately upstream of *amiY*. However, the mapping of a 3' end of a transcript would produce identically sized S1 nuclease products with both probes yet mapping the 5' end of a transcript would produce different sized S1 products with each probe. In this way it would be possible not only to map the position of the termination site immediately downstream of *amiE* but it would also be possible to detect a separate *amiY* transcript.

A further advantage of this experiment is that it would demonstrate how much read through transcription occurred at this potential terminator site since read through transcripts would fully protect both probes. A danger with this type of analysis is that it might not be possible to tell whether the S1 nuclease digestion had gone to completion but fortunately the probes were synthesised from M13 substrates, using universal primer, so they contained a small region of M13 DNA which would not hybridise with the RNA therefore would not be protected from S1 nuclease digestion. Therefore, if the S1 nuclease digestion has gone to completion the non-homologous M13 DNA sequence should be removed and a probe fully protected by read through transcripts should be slightly smaller than the original probe.

The two probes described above would be expected to produce large S1 nuclease protection products (approximately 300bp) when mapping the 3' end of an *amiE* transcript. Thus a third probe was also employed in this analysis which would produce much smaller S1 nuclease resistant products which could be accurately resolved on a sequencing gel. This would allow fine mapping of the transcription termination point immediately downstream of *amiE*.

The two probes used for the first part of the experiment were synthesised from the M13 clones SAW14 and SAW16 (see Section 6.4.1.). Figure 6.14 illustrates the probes used for this experiment. SAW14 contains the 1.4kb *Hin*dIII-*Hin*fI fragment from pAS20 subcloned into the *Sma*I target of M13mp18 and SAW16 contains the 1.4kb *Ban*II fragment from pAS20 subcloned into *Sma*I cut M13mp18. The continuously labelled probes were synthesised using universal primer as described in

materials and methods, and the single stranded probes were released from the M13 templates by cleavage with *Pvu*II . This produced two probes with an identical 5' end mapping to the *Pvu*II site within the *amiE* gene but one probe had a 3' end mapping to the *Hinf*I target at position 1458 giving a probe size of 482bp including 57bp of M13 sequence. The second probe had a 3' end mapping to the *Ban*II target at position 1722 and had a size of 746bp including 57bp of M13 sequences. DNA sequencing ladders were used to provide suitable size markers in all S1 mapping experiments.

The first S1 mapping experiment using the two large probes described above was carried out using induced RNA from PAC1, PAC200 and PAC452 pSW101. Since both the probes and the S1 products were expected to be large it would not be possible to accurately resolve bands on a sequencing gel and this first experiment was designed to roughly indicate where the 3' end of the transcript might be and to what extent read through transcription occurred. Figure 6.15 shows the results obtained.

Lane 1 contains the *Pvu*II-*Hinf* I probe alone and shows a strong band with an estimated size of 480bp which agrees closely with the predicted size of 482bp. There is a significant amount of degradation of the free probe since it was continuously labelled and is therefore subject to random radiochemical degradation between the time of preparation and use. This degradation could not be avoided but was kept to a minimum by using the probes within 15 hours of their synthesis. Lane 2 contains the results of the S1 analysis with RNA isolated from an induced culture of PAC1. Major bands appear at the following approximate sizes, 420bp, 325bp, 317bp, 313bp, 255bp, 145bp and 128bp. The top band with a predicted size of 420bp corresponds to the length of the probe used minus the non-homologous M13 DNA sequences (should =425bp). Some of these bands are quite faint and can be seen more clearly in lanes 3 and 4.

The presence of the 420bp band indicates two things. Firstly, the S1 nuclease digestion has gone to completion since no band is seen at a size corresponding to the free probe, and consequently this band indicates that transcripts are present

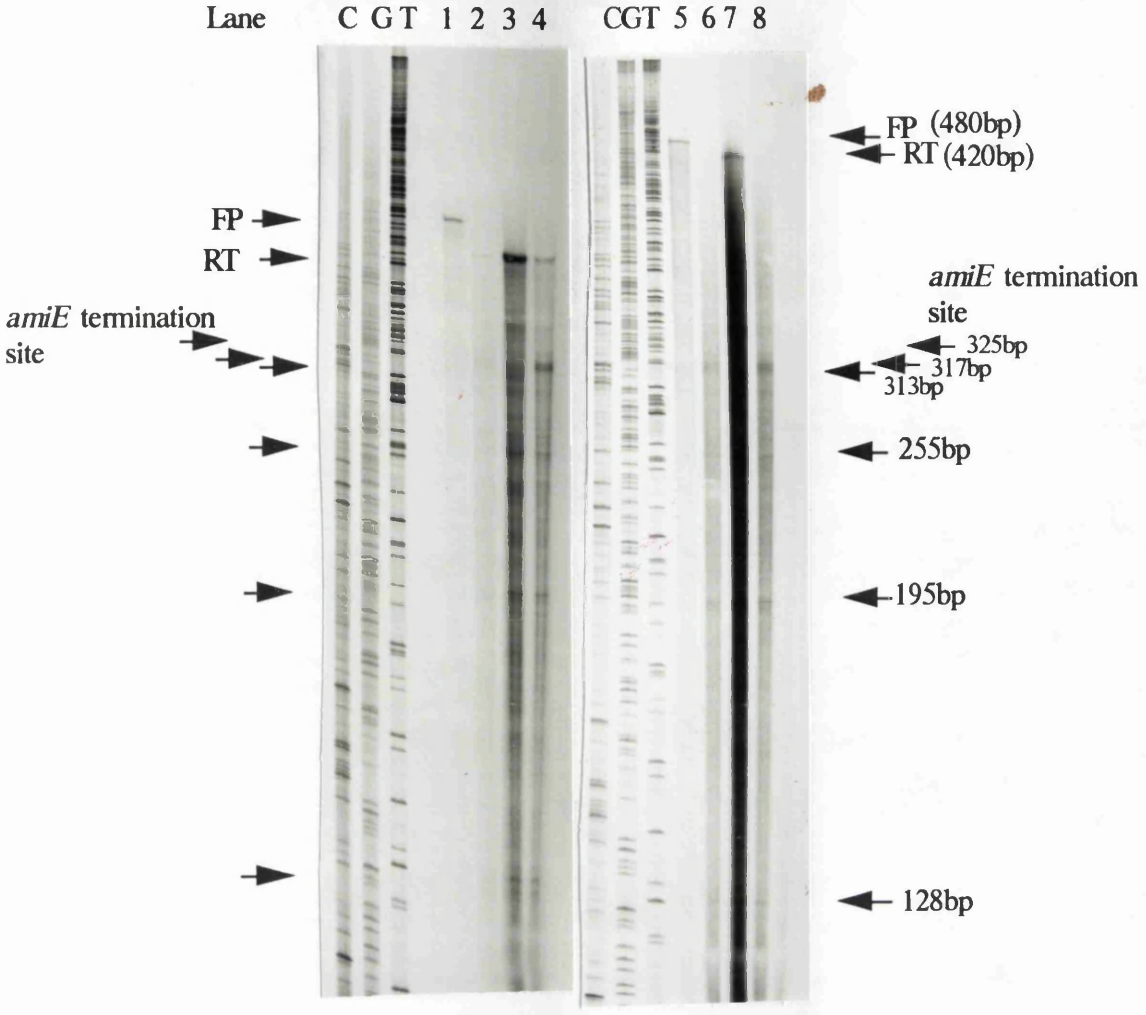


Figure 6.15. S1 nuclease mapping of the 3' end of the *amiE* transcript using continuously labelled probes. Lanes labelled C-G-T correspond to sequencing ladders used as size markers. Samples were loaded as follows: Lane 1, undigested *Pvu* II- *Hinf*I probe; lane 2, PAC1 induced RNA; lane 3, PAC452 pSW101 induced RNA; lane 4, PAC200, induced RNA; lane 5, undigested *Pvu* II-*Ban* II probe; lane 6, PAC1 induced RNA; lane 7, PAC452 pSW101 induced RNA; lane 8, PAC200 induced RNA. Each reaction used 20 microgrammes of RNA. FP = free probe, RT= protected probe (-M13 sequences), corresponding to read through transcripts. The tightly clustered triplet of bands marked *amiE* termination site map immediately downstream of the 3' *amiE* terminator. The three smaller S1 products marked by the arrows, probably represent artefactual bands produced by A-T rich regions in the DNA-RNA hybrid, susceptable to S1 nuclease atttack. The appoximate sizes of bands are indicated alongside the arrows.

which fully protect the probe which much extend at least down to the *Hinf*I target at position 1458. This confirms that read through transcripts are produced bigger than the discrete *amiE* transcript of 1.3kb. The three closely clustered bands with sizes of 325bp, 317bp and 313bp map just 3' of the axis of dyad symmetry downstream of *amiE* (see figure 6.17). These bands probably represent the site of termination of transcripts which only encode for *amiE* (i.e 1.3kb transcripts).

There are three other major bands with sizes of 255bp, 195bp and 128bp. If these bands represented 3' ends of transcripts they would map within the *amiE* gene. However, if these bands represented 5' ends of transcripts they would be expected to appear as different sized bands with the larger *Pvu*II-*BanII* probe however looking at the results with this larger probe (lanes 6,7,8), these bands also appear at similar positions. It seems most likely that these bands represent regions of the DNA-RNA hybrid which are possibly AT rich and are susceptable to non-specific cleavage by the S1 nuclease. The RNA used for the reaction in lane 3 was from PAC452 pSW101 and shows a pattern of bands which is very similar to that seen in lane 2 with the PAC1 RNA, although clearly there are more transcripts present so more of the probe was protected from S1 nuclease digestion. This was expected since PAC452 pSW101 produces up to 50 units of amidase compared with an average of 5 units produced by induced PAC1. The bands which map 3' of the potential terminator downstream of *amiE* are not as clear with this sample which could suggest more read through transcription is occurring. Lane 4 contains RNA isolated from PAC200, the *ntrA*$^-$ mutant of PAC1 which shows high induced amidase levels. This also produces an similar pattern of bands to that seen with induced PAC1 RNA. The cluster of three bands which map 3' of the potential transcription terminator downstream of *amiE* show up very clearly in this lane.

Lane 5 contains the *Pvu*II–*Ban*II probe alone and shows a band with a calculated size of 750bp. Once again a smear corresponding to radiochemical cleavage of the probe is seen. Lane 6 contains RNA from PAC1 induced, lane 7 ; RNA from PAC452 pSW101 induced and lane 8 RNA from PAC200 induced. All three of these lanes show a band which has a predicted size of 700bp which corresponds to

fully protected probe minus the non-homologous M13 DNA sequences. These bands differ in intensity in a way which related directly to how much amidase is produced by the strain. The autoradiograph shows that read through transcripts are produced which extend beyond the potential terminator 3' of *amiE* and also extend beyond the *BanII* target at position 1722. All three lanes also show the triplet of bands of sizes 313bp, 317bp and 325bp which map 3' of the potential transcription terminator downstream of *amiE* They also show the smaller S1 products also seen with the *Pvu*II-*Hinf*I which probably represent artefactual cleavage by S1 nuclease at AT rich sequences.

Since both probes produced identical banding patterns this would suggest that there are no 5' ends of transcripts starting between the 3' end of the *amiE* gene and the 5' end of the *amiY* gene. Secondly, since both probes produce bands which correspond to the fully protected probes this shows that the transcription termination site 3' of *amiE* can be read through to produce transcripts which encode all of the amidase genes. This experiment has identified the 3' ends of the *amiE* transcripts. The presence of 3' ends shows that the axis of dyad symmetry downstream of *amiE* can act as a transcription terminator. It is difficult to assess the percentage of transcript read through at this termination site but it is estimated at 40% of transcripts. The absence of any 5' ends of transcripts upstream of the *amiY* start codon indicate that this gene is only transcribed by read through of the *amiE* terminator.

The mapping of the 3' ends of transcripts which stop close to the potential axis of dyad symmetry was rather crude in this experiment and so a second S1 mapping experiment was carried out using the two probes described above and a third probe which would produce much shorter S1 products. The third continuously labelled probe was made in the same way as the two probes described above using sequencing clone SAW13. This clone contains the 156bp *Nae*I fragment from pAS20 subcloned into M13mp18. This *NaeI* fragment straddles the *amiE* transcription termination region and is illustrated in Figure 6.14. The probe was constructed using universal primer and was separated from the M13 template by

digestion with *Eco*RI. The probe thus contains both 5' and 3' sequences derived from M13 which are non-homologous with the RNA and will be digested away by the S1 nuclease. The *Nae*I probe had a size of 230bp including 76bp of M13 sequence and was used along with the two probes described previously with RNA isolated from PAC1 non-induced and induced and PAC200 non-induced and induced cultures and the results are shown in Figure 6.16.

The results with the *Pvu*II-*Hinf*I probe and the *Pvu*II-*Ban*II probe are essentially the same as shown previously in Figure 6.15. Some probe being fully protected and some probe giving a triplet of bands mapping to the proposed transcription terminator downstream of *amiE*. Some non-induced RNA samples were used with the *Ban* II probe and as expected, these do not protect the probe from S1 nuclease digestion. Lane 13 contains the *Nae*I probe alone and shows a major band with a size of 230bp. There has been substantial radiochemical degradation of the probe between isolation and use, producing a ladder of smaller, less intense bands. Lane 9 contains the *Nae*I probe incubated with non-induced PAC1 RNA and shows no protected products. A similar results is seen in Lane 11 with non-induced PAC200 RNA. Lanes 10 and 12 contain the samples produced by incubation with PAC1 and PAC200 induced RNA respectively. Both lanes show a major band of 156bp which corresponds to fully protected probe minus the M13 sequences. There are also numerous bands which correspond to radiochemical cleavage products of the probe. In addition, there is an intense cluster of bands shown by the arrows which are likely to represent the 3' end of *amiE* transcripts (marked S1 in Figure 6.16). The cluster of bands appears to fall into three discreet regions, closely spaced which correspond to the triplet of bands seen with the larger probes. The positions that these bands map to is shown in Figure 6.17. Two other major bands are seen below the cluster (S2 and S3) and the positions they map to are shown in Figure 6.17.

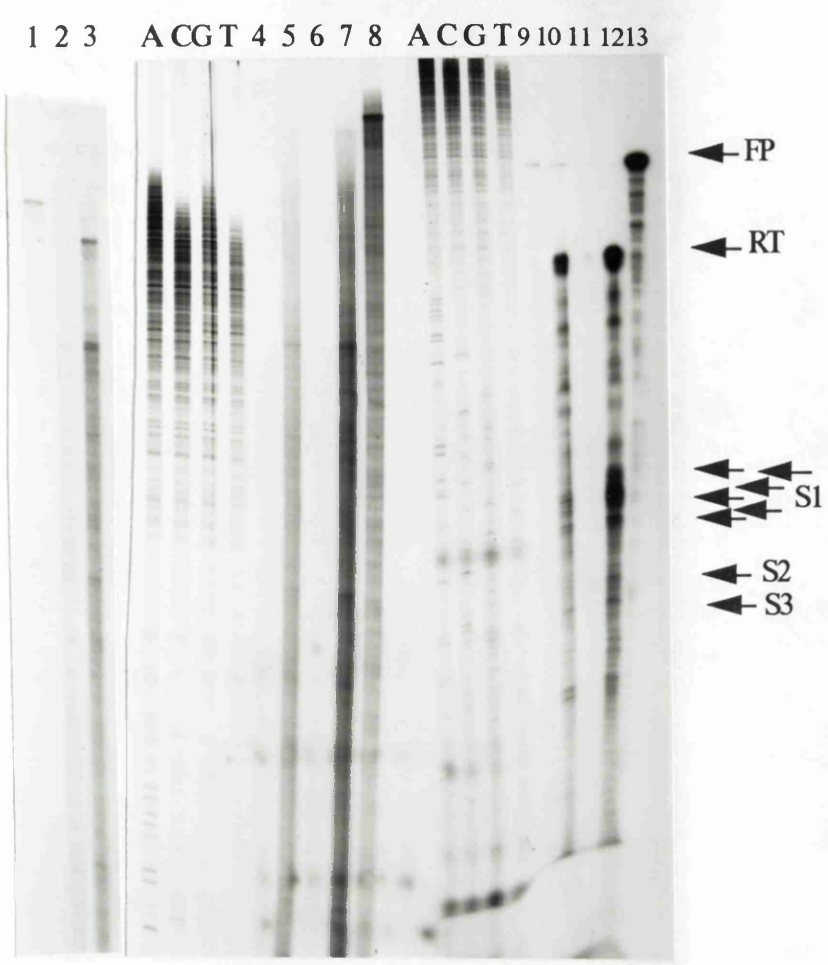


Figure 6.16. S1 nuclease mapping of the 3' end of the *amiE* transcript using continuously labelled probes. Lanes labelled A-C-G-T correspond to sequencing ladders used as size markers. Samples were loaded as follows: Lane 1, undigested *Pvu* II- *Hinf* I(P-H) probe; lane 2, PAC1 induced RNA, (P-H probe); lane 3, PAC200, induced RNA (P-H probe); lane 4, PAC1 non-induced RNA, *Pvu* II-*Ban* II (P-B probe); lane 5, PAC1 induced RNA (P-B probe); lane 6, PAC200, non-induced RNA (P-B probe); lane 7, PAC200 induced RNA, (P-B probe); lane 8, undigested P-B probe; lane 9, non-induced PAC1 RNA, *Nae*I (N) probe; lane 10, PAC1 induced RNA (N probe) ; lane 11, PAC200 non-induced RNA (N-probe); lane 12, PAC200 induced RNA (N-probe); lane 13, free, undigested N-probe.Each reaction used 20 microgrammes of RNA. FP = free probe, RT= protected probe (-M13 sequences). The major S1 products are indicated by the arrows and labelled S1-S3. The positions of these major bands are shown with the DNA sequence in Figure 6.17.

To estimate the percentage of transcripts which read through the terminator 3' of *amiE*, scanning densitometry was performed on lane 12 in Figure 6.16 which contained induced RNA from PAC200. The results of this analysis are shown in Figure 6.18. By addition of the peak integrals corresponding to terminated transcripts and comparing this with the peak integral for read through transcripts it was estimated that 40% of transcripts read through this terminator.

Whether this read through is AmiR dependent cannot be established from these experiments. However the motifs which are thought to be involved in the antitermination process upstream of the regulated terminators for amidase and the *bgl* operon (Drew and Lowe, 1989), are absent upstream of this 3' terminator.

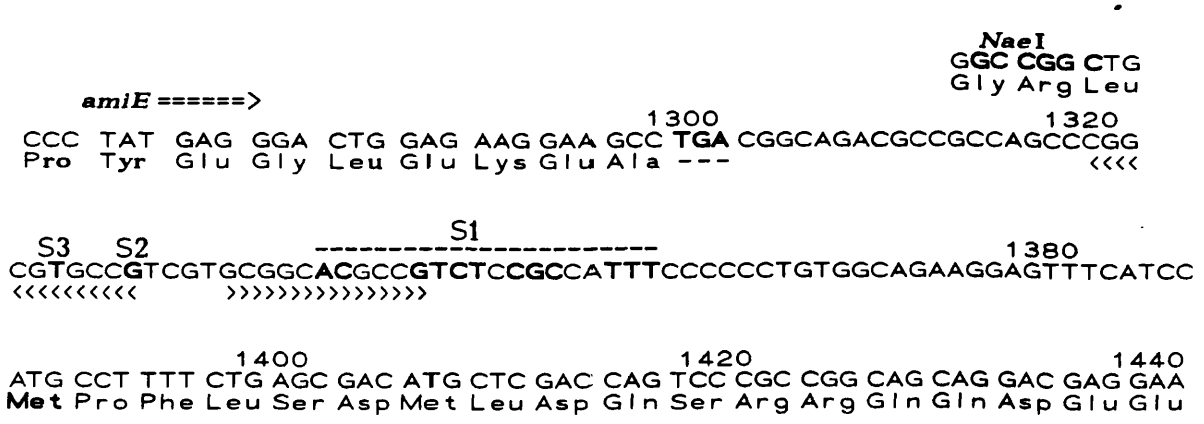
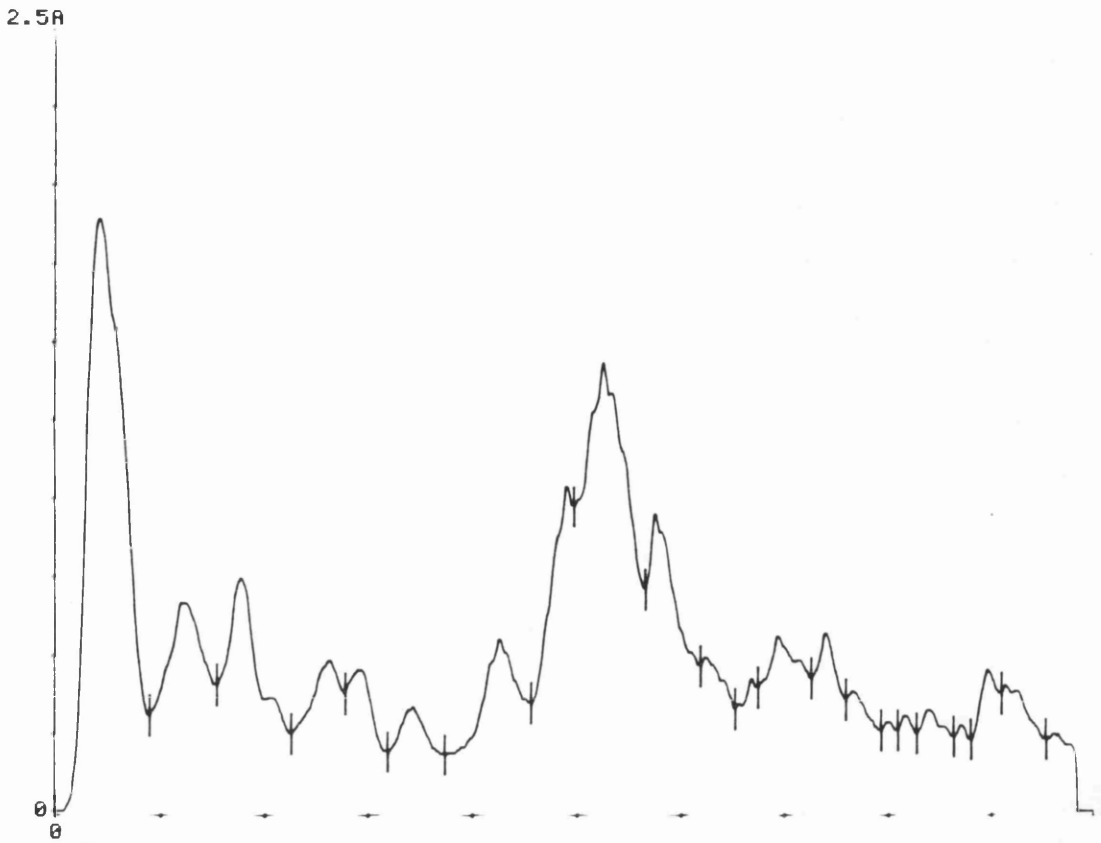


Figure 6.17. Location of the transcription termination sites 3' of the *amiE* gene. The C-terminal sequence of the *amiE* gene is shown, as is the N-terminal sequence of the *amiY* gene. The axis of dyad symmetry 3' of the *amiE* gene is identified by <<< >>>> symbols. Points at which transcription terminates (S1, S2 and S3) which were identified by high resolution mapping in Figure 6.16 are identified by the bold sequence. The low resolution mapping in Figure 6.15 also indicated termination sites mapped immediately downstream of the axis of dyad symmetry.



LENGTH OF X-AXIS= 170 MM.

TOT. INTEGRAL= 174544

PEAK	POSITION	HEIGHT	REL%	INTEGRAL	PEAK	POSITION	HEIGHT	REL%	INTEGRAL
1	7.4	195	16.32	28496	2	21.2	69	6.56	11460
3	30.6	77	6.7	11708	4	45.2	50	3.93	6864
5	50.2	47	2.91	5084	6	59.0	35	2.77	4852
7	72.8	57	5.86	10232	8	83.6	107	5.87	10256
9	89.8	147	15.27	26660	10	98.0	98	7.68	13416
11	106.8	51	2.88	5044	12	114.2	44	1.67	2932
13	118.4	58	4.97	8684	14	126.4	59	3.1	5416
15	131.0	40	2.27	3968	16	136.8	30	0.91	1600
17	139.6	32	1.01	1776	18	143.4	34	2.01	3516
19	148.6	29	0.85	1484	20	153.0	47	2.27	3972
21	156.0	42	2.77	4844	22	163.8	26	1.3	2280

Figure 6.18. Scanning densitometry analysis to determine the percentage of read through transcription at the terminator downstream of *amiE*. Lane 12 shown in fig. 6.16. was scanned using a Joyce Loebl chromoscan 3. The first peak corresponds to read through transcripts. Peaks 7,8,9,10,13 and 14 correspond to transcripts terminating at the transcription terminator downstream of *amiE*. By addition of the peak integrals corresponding to terminated transcripts and comparing this with the peak integral for read through transcripts, it was estimated that 40% of transcripts read through the transcription terminator.

In conclusion these experiments have demonstrated three important points. Firstly the axis of dyad symmetry downstream of *amiE* can terminate transcription giving a 1.3kb transcript which encodes only *amiE*. Secondly, 40% of transcripts originating from the *amiE* promoter read through this terminator as suggested by the Northern analysis and go on to produce full length transcripts of approximately 5.0kb which encode *amiE*, *amiY*, *amiC*, *amiR* and *amiX*. Finally there does not appear to be a separate promoter for *amiY* . Therefore this gene can only be expressed by read through transcription.

6.9 Mapping transcription starts originating from the *ntrA* dependent promoters

Previous experiments described in this chapter have shown that transcription from the *amiE* promoter under inducing conditions can read through the transcription terminator and continue to produce a transcript of approximately 5.0kb. The next set of experiments were designed to establish the role of the *ntrA* dependent promoters by identifying possible 5' ends of transcripts using primer extension analysis and S1 nuclease mapping. This approach is fraught with potential difficulties because of the read through transcription of the *amiE* promoter. However an oligonucleotide was synthesised which was complementary to the 5' end of the *amiC* gene (oligoC) in order to carry out these experiments. The sequence of this oligonucleotide and the complementary DNA sequence is shown in Figure 6.20.

A combination of S1 nuclease analysis and primer extension analysis are often used to map the 5' ends of transcripts since each technique has inherent problems (Sambrook *et al*, 1989). Since oligoC had been synthesised this could be used to make an S1 nuclease probe which would start at the same point as a primer extension.

Thus the 5' end of a transcript would produce identical sized products with both techniques, allowing a comparison on the same sequencing gel. Furthermore, oligoC could also be used as a primer in a sequencing reaction. Thus the mapping of a product to a particular position could be achieved simply by reading the sequence at the position of the product.

The primer extensions and sequencing reactions with the oligoC were carried out as described in materials and methods. The S1 probe was prepared using end-labelled oligoC and sequencing clone SAW2. The probe was removed from the template by cleavage with *Xho*I, producing a 155bp probe. Following the S1 digestion and primer extension reactions, all samples were loaded on an 8% polyacrylamide-urea sequencing gel and the results are shown in Figure 6.19 with a variety of RNA samples.

The critical test of whether or not the *ntrA*-dependent promoters identified within the 658bp *Xho*I fragment are functional was to try and map the 5' ends of transcripts using the techniques described above, from a strain which has the potential to use these promoters i.e. an *ntrA*$^+$ strain and from a strain lacking this sigma factor which should be unable to use these promoter elements.

Firstly considering the primer extension experiments shown in lanes 9-15, a bewildering array of extension products are seen. The major extension products are indicated by arrows and labelled. The positions they map to in the sequence upstream of *amiC* are shown in Figure 6.20. The important point to consider from this analysis is the pattern of bands seen with JA221 pJB950 grown in the absence of nitrogen (lane 14) and ET8045 pJB950 (lane 15) since these represent *ntrA*$^+$ and *ntrA*$^-$ backgrounds respectively and the *ntrA* dependent promoters should be activated under nitrogen limiting conditions in JA221. There is no evidence for any bands which map to the appropriate positions downstream of the *ntrA*-dependent promoters which are present with JA221 pJB950 (-N) and absent from ET8045 pJB950. In fact the pattern of bands seen in both lanes is essentially identical. This is an important point since it means that none of the bands seen can represent 5' ends of transcripts which originate from the potential

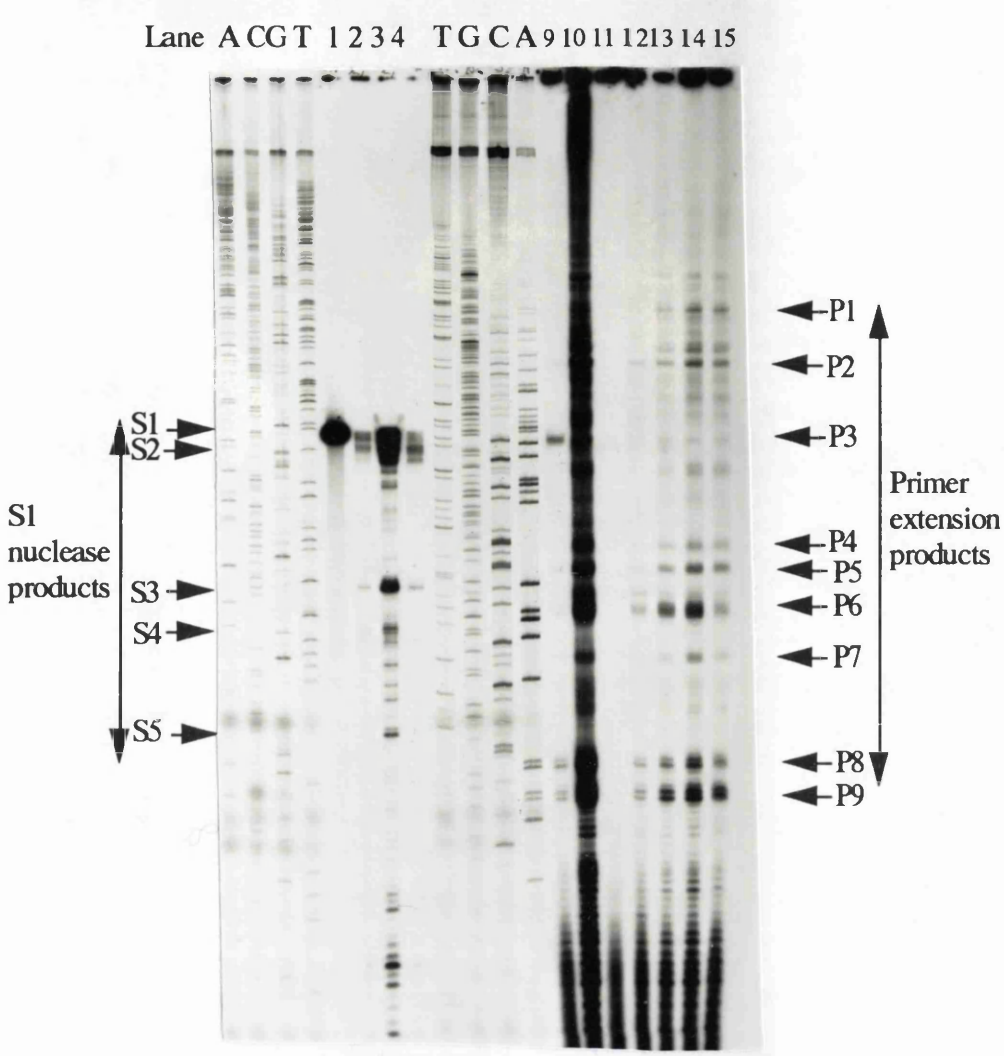


Figure 6.19. S1 nuclease and primer extension analysis on the two *ntrA* dependent promoter elements upstream of *amiC*. Samples were loaded as follows: lane 1, undigested *Xho*I-oligoC (X-O) S1 probe; lane 2, PAC1 induced RNA (X-O probe); lane 3, PAC452 pSW101 induced RNA (X-O probe); lane 4, PAC452 pSW101 non-induced RNA (X-O probe); lane 5,,6,7 and 8, sequencing reaction using oligo C, giving the DNA sequence upstream of *amiC*; lane 9-15 primer extension reactions loaded as follows; lane 9, PAC452 pSW101 non-induced RNA; lane 10, PAC452 pSW101 induced RNA, lane 11, PAC1 non-induced RNA; lane 12, PAC1 induced RNA, lane 13, JA221 pJB950 nitrogen excess RNA; lane 14, JA221 pJB950 nitrogen limiting RNA, lane 15, ET8045 pJB950 nitrogen excess RNA. The major primer extension products and S1 nuclease products are indicated with arrows. All reactions used 20 microgrammes of RNA except ET8045 pJB950, (lane 15), which used 10 microgrammes.

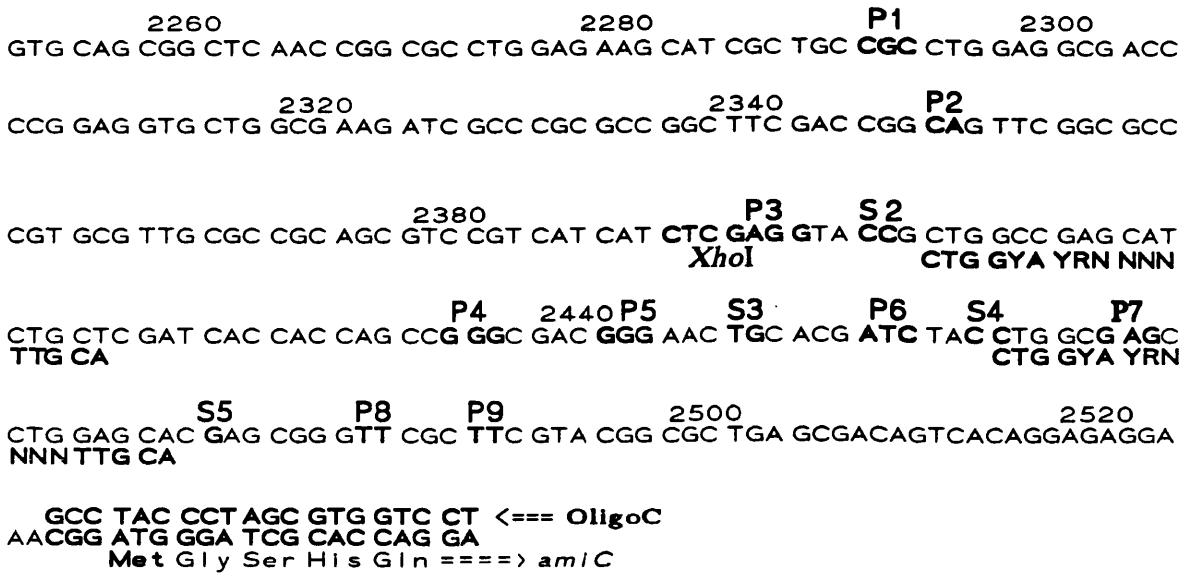


Figure 6.20. Location of oligoC and tne primer extension products and S1 nuclease products using this oligonucleotide. The sequence is numbered from the unique *Hin*dIII target upstream of *amiE*. The TGA sequence at positions 2500-2502 corresponds to the stop codon for *amiY*. The start of the *amiC* coding sequence is shown. The sequence of oligoC is shown above its complement. The consensus σ^{54}-dependent promoter sequence is shown in bold below the two sequence motifs with homology to this consensus. The positions of the extension products P1-P9 identified in Figure 6.19 are indicated in bold. Similarly, the positions of the S1 nuclease products S2-S5 are shown in bold.

ntrA-dependent promoters. Furthermore, lane 14 containing RNA isolated from JA221 pJB950 grown under nitrogen limiting conditions shows no major new bands, compared with lane 13, containing JA221 pJB950 grown under nitrogen excess growth conditions.

Since at least some transcripts covering this region will have originated from the *amiE* promoter it would seem possible that the ladder of bands seen in lanes 13-15 corresponds to prematurely terminated extension products which would ordinarily extend all the way to the *amiE* promoter. Such bands could be produced by degradation of full length transcripts, which the Northern analysis indicated or possibly some bands could be produced by regions of secondary structure in the RNA which the reverse transcriptase has difficulty reading through.

There is however, *in vivo* evidence for promoter activity in the DNA sequence between the *Xho*I target(2400) upstream of *amiC* and the 5' end of *amiC* (Cousens *et al*, 1987; this study). Therefore one of the extension products (P4-P9 in Figure 6.20) seen in lanes 13-15 could correspond to the 5' end of a transcript originating from a promoter in this region of DNA. However, it is not possible to tell which extension product this is.

Lanes 9 and 10 show the primer extension products produced with RNA isolated PAC452 pSW101 non-induced and induced respectively. The induced RNA in lane 12 shows an intense ladder of extension products which run to the top of the gel, confirming read through from the *amiE* promoter occurs under inducing conditions. Interestingly the non-induced RNA in Lane 9 shows an extension product (P3) which maps immediately downstream of the *Xho*I target upstream of *amiC* (see Fig. 6.20). The amount of read through transcription in a non-induced culture should be quite low and yet this is a major extension product. This band is also seen very faintly in Lane 11 which contains non-induced PAC1 RNA. It is tempting to speculate that this extension product (P3) corresponds to the promoter activity known to exist within the 658bp *Xho*I fragment but this seems unlikely since the transcription start point would only be 2bp away from the *Xho*I target and thus the recognition sequences for RNA polymerase binding would lie

outside this fragment.

The results of the S1 nuclease analysis are shown in Lanes 1-4. The major S1 nuclease products are labelled S1 to S5 and the positions to which they map are shown in figure 6.20. Lane 1 contains the free probe (S1) which maps to the *Xho*I target in the DNA sequencing ladder as expected. Lane 2 contains RNA isolated from an induced culture of PAC1, and shows a substantial amount of the probe is fully protected, though there are some bands with a size slightly smaller than full length (S2) which could represent slight over digestion with the S1 nuclease, since these bands map too close to the *Xho*I target to represent the promoter activity within the 658bp *Xho*I fragment. However, the S1 nuclease product S2 and the primer extension product P3 both map to approximately the same position and this could represent promoter activity originating from a site immediately upstream of the 658bp *Xho*I fragment.

A similar pattern of bands is seen in lanes 3 and 4 which contain induced and non-induced PAC452 pSW101 RNA respectively, though the intensity of bands is greater with the induced culture. The major band S3, seen with all samples does not seem to have a corresponding extension product in the primer extension reactions in lanes 9-15 indicating this could be an artefactual band, produced by nicking of the probe by S1 nuclease due to breathing of the DNA-RNA hybrid. Alternatively if S1 nuclease over digestion had occurred this band might correspond with the primer extension product P5. However, this transcription start point maps between the two possible ntrA dependent promoter elements. Thus whilst it would be sufficiently far downstream into 658bp *Xho*I fragment, it is not preceded by any recognisable consensus promoter sequences. There are two other major S1 nuclease products S4 and S5 (see Fig. 6.20 for positions) and one of these bands might represent the transcription start point for the promoter within the 658bp *Xho*I fragment.

In conclusion the primer extension analysis and S1 nuclease analysis have shown that there are no major transcripts which originate from the *ntrA* dependent promoters. The role of these sequences thus appears to be to down regulate *amiCRX* expression. These experiments have not however excluded the

possibility of promoter activity within the 658bp *Xho*I fragment since a large number of primer extension products and S1 nuclease products have been identified. However, in the absence of alternative recognisable consensus promoter sequences it is impossible to tell which, if any, of these bands represent true 5' ends of transcripts.

Much confusion could have been avoided in this analysis if the *amiE* promoter was removed by construction of a suitable new plasmid. This would then have removed the read through transcripts, which simply confused the analysis and confounded the analyser! Time did not allow such a plasmid to be constructed.

6.10 Summary

The DNA sequence analysis has identified two new genes; *amiY* which lies between *amiE* and *amiC* and *amiX* which lies downstream of *amiR*. The function of these two genes in amidase regulation remains unclear, though *amiY* clearly contains a nucleotide binding domain.

The nature and function of the promoter activity within the 658bp *Xho*I fragment has remained elusive. The *in vivo* evidence for promoter activity is as follows:

1. Plasmid pDC44 which contains pJB950 DNA from the *Kpn*I target at position 2400 to the *Sal*I target at position 5400 subcloned into pKT231 is capable of complementing the *amiR*$^-$ strain PAC327 (Cousens, 1985).

2. Inversion of the 658bp *Xho*I fragment in pSW1 causes a reduction in amidase expression, suggesting that convergent transcription in addition to *ntrA* mediated repression prevents read through transcription from the *amiE* promoter and also prevents any transcription from the promoter element in the 658bp *Xho*I fragment.

3. pSW12 which contains the 658bp *Xho*I fragment subcloned upstream of a promoterless *amiE* gene shows a low constitutive level of amidase expression.

The immediately obvious promoter elements within this region of DNA are the two *ntrA* dependent promoter sequences. *In vivo* it has been shown that *ntrC* can activate amidase expression and this activation is dependent on the presence of these promoter elements. However it has been found by Cousens *et al*, (1987) that small deletions which encompass these potential promoter elements lead to increased amidase expression. For example pDC11 which has a 170bp bidirectional deletion from the unique *Kpn*I target in pJB950, produces 11 units of amidase activity compared to 4 units for pJB950 (Cousens *et al*, 1987). A similar result has been obtained with pSW3 and pSW5 described in Chapter 3. This result was confusing since it was expected that deletion of these promoter elements would lead to a reduction in amidase expression.

Subsequently it was shown that *ntrA* inactivation, in both *E.coli* and *P.aeruginosa* caused similar increases in amidase expression to that seen when i) the promoter elements are deleted, ii) the promoter elements are activated by NtrC. These findings led to the hypothesis that an NtrA-holoenzyme closed complex, bound to either or both of the *ntrA* dependent promoters was capable of blocking transcription from the upstream *amiE* promoter.

The primer extension analysis described in Section 6.7 mapped the transcription start point for the *amiE* promoter and also confirmed that transcription from this promoter was constitutive. The transcript analysis showed that transcription from the *amiE* promoter under inducing conditions, can either stop at the transcription terminator downstream of *amiE* producing a 1.3kb transcript, or in approximately 40% of cases, can proceed through this terminator and continue through the entire operon, terminating 3' of *amiX*. No 5' ends of transcripts were identified immediately upstream of the *amiY* gene indicating that this gene can only be transcribed by read through of the 3' *amiE* terminator. If the proposal that the *ntrA* dependent promoter elements represent a site for transcription termination is correct then the Northern analysis should have revealed appropriately sized transcripts, yet none were found. However, the results were confused by degradation of full length transcripts. Consequently the possibility still remains that transcripts do terminate at this point in some cases. Of course it is difficult to

estimate what percentage of transcripts might normally terminate at these promoters, since a small increase in *amiCR* transcription might lead to a large increase in amidase expression.

The Northern analysis also failed to identify any transcripts which originated from the promoter activity in the 658bp *Xho*I fragment. Once again the results were largely confused by degradation of full length transcripts. Therefore in a final attempt to identify the promoter activity in this region of DNA the primer extension analysis and S1 nuclease analysis was carried out with oligoC. This analysis showed no evidence for transcripts which were specific for an *ntrA*$^+$ host grown under nitrogen limiting conditions, which confirmed that the *ntrA*-dependent promoter elements are not responsible for the promoter activity in the 658bp *Xho*I fragment. This finding provided further evidence for these promoter-like sequences representing a negative control system as described above with NtrA-holoenzyme. The analysis did not exclude the possibility of promoter activity within the small *Xho*I fragment though since a large number of primer extension products and S1 products were seen. However, it was impossible to tell which of these represented a true 5' end of a transcript.

In conclusion it would appear from the *in vivo* evidence that there is some weak (non-*ntrA* dependent) promoter activity within the 658bp *Xho*I fragment and this probably serves to provide background constitutive synthesis of the negative regulator, *amiC* and the positive regulator *amiR*. Upon induction this pair of proteins are thought to respond to inducer allowing functional AmiR to cause antitermination at the *amiE* promoter.

Transcripts which originate from the *amiE* promoter can have several fates as follows. Firstly they can terminate at the transcription terminator upstream of *amiE* under non-inducing conditions. Secondly, under inducing conditions, they can terminate immediately downstream of *amiE* producing a 1.3kb transcript. A proportion of these transcripts read through this termination site and proceed through *amiY*. It would then appear that the transcripts meet a second barrier which are the two *ntrA* dependent promoter elements upstream of *amiE*. It seems likely that some transcripts terminate at this site and this termination process

might normally be regulated by nitrogen availability via an NtrC homologue in *P.aeruginosa*. This is discussed in more detail in Chapter seven. Some transcripts always read through this second barrier thus allowing expression of *amiC*, *amiR*, and *amiX* since full length transcripts are seen in ammonia excess growth conditions. Therefore the terminator downstream of *amiE* and the potential termination site 5' of *amiC* provide a gradient of transcripts originating from the *amiE* promoter.

In terms of constructing a model for the amidase induction process, the most important point to come out of these experiments is that under inducing conditions, the majority of *amiC*, *amiR* and *amiX* transcription appears to originate from the *amiE* promoter. Since *amiR* regulates the activity of the *amiE* promoter it would appear that *amiR* regulates its own expression in conjunction with *amiC*. These findings are discussed further in Chapter seven.

CHAPTER SEVEN - DISCUSSION

At the start of this project, amidase genes had been cloned from strains PAC1 (wild type) and PAC433 (constitutive mutant) (Wilson and Drew, 1991; Drew *et al*, 1980). The location, direction of transcription, DNA sequence and amino acid sequence of *amiE* (amidase) had been determined (Brammar *et al*, 1987; Ambler *et al*, 1987). In addition the location, direction of transcription and DNA sequence of the PAC433 *amiR* gene had also been determined (Cousens *et al*, 1987; Lowe *et al*, 1989). DNA sequencing studies of the *amiE* leader region from PAC1 and PAC433 had indicated that *amiE* was transcribed from an *E.coli*-like promoter. Plasmid reconstruction experiments had shown that amidase expression was positively regulated by a transcription antitermination mechanism, mediated by AmiR (Drew and Lowe, 1989).

In addition to the studies at UCL using PAC strains, the amidase system of *P.aeruginosa* PAO1 has been investigated using the mutator phage D3112 (Rehmat and Shapiro, 1983). Selection was imposed for Ami$^-$ mutants and the sites of insertion of D3112 in these mutants were identified by Southern blotting. Ami$^-$ insertions were identified in three locations (Figure 7.1). One group of Ami$^-$ mutants were located to the *amiE* region, one group to the *amiR* region and a third group defined as leaky mutants were located between *amiE* and *amiR*. From the current study it appears that these insertions lie within *amiY*.

At this time, the induction process and the role of inducing amides had not been characterised. The results presented in this thesis are sufficient to justify the construction of a model to explain amidase regulation. The construction of the model will be broken down into three separate stages as follows: i) The function of AmiC and AmiR, ii) the transcriptional regulation of the whole operon and iii) the function of AmiY. These related discussions will be brought together to give the final model.

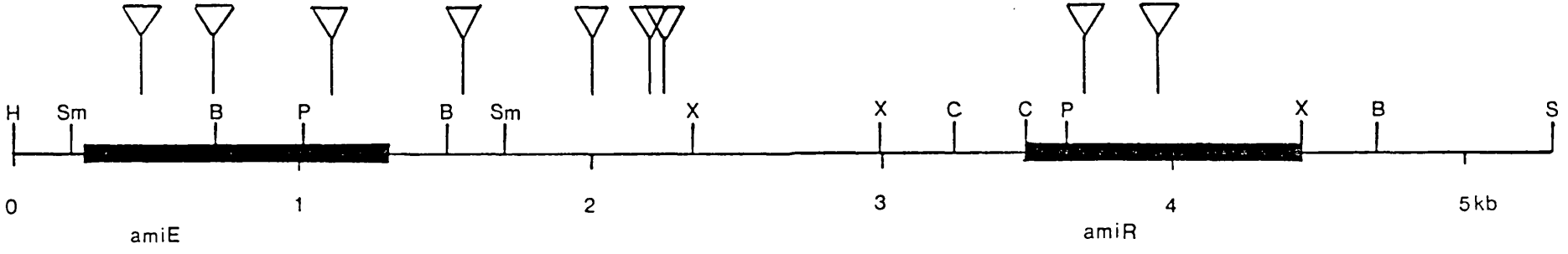


Fig. 7.1 Location of D3112 prophages in the ami region of P. aeruginosa PAO1.

Data from Rehmat and Shapiro (1983).

Abbreviations: HindIII (H), SmaI (Sm), BalI (B), PvuII (P), XhoI (X), ClaI (C), SalI (S); amidase structural gene (amiE); amidase regulator gene (amiR); location of D3112 prophage (▽).

1) **The function of AmiC and AmiR**

In Chapter three, a series of plasmid constructs were made which showed that AmiR functioned independently of amide inducers. These included the *amiC* insertion and deletion mutants pSW36 and pSW37, and the transcomplementation system pDC5, pSW35. All of these showed constitutive amidase expression mediated by the wild type *amiR* gene. The high level of amidase expression seen with pDC5 pSW35, in which *amiR* was transcribed from a strong vector promoter, showed there was a correlation between the level of AmiR and the level of amidase. The transcomplementation system with the broad host range *amiR* expression vector, pSW40, in PAC1, showed it was possible to provide saturating amounts of AmiR *in trans.*

AmiC is a negative regulator of amidase expression. This was demonstrated by showing that a deletion and frameshift mutation within the *amiC* gene (pSW36, pSW37) rendered the wild type inducible genes in pAS20 constitutive. This was further confirmed by the introduction of the broad host range *amiC* expression vector, pSW41, into amidase constitutive *P.aeruginosa* strains and showing repression of amidase synthesis.

Subsequently it was shown that AmiC repressed the antitermination activity of AmiR by a protein-protein interaction. This was demonstrated using *amiE*, *amiR* and *amiC* on three separate plasmids in *E.coli* as follows: The *amiR* gene was constitutively transcribed from the *lac* promoter, and transcomplementation with the *amiR* and *amiE* plasmids showed high levels of amidase activity. The introduction of the *amiC* expression vector into the system, caused repression of amidase expression. Since AmiC would be unable to affect transcription of *amiR* from the *lac* promoter, this confirmed that AmiC repression of AmiR antitermination activity was at the protein level.

Since BglF repression of BglG was mediated by phosphorylation (Amster-Choder *et al*, 1989), it was decided to test whether AmiC worked in a similar manner, despite a lack of homology between AmiC/BglF and AmiR/BglG. Using purified AmiC, γ-^{32}P-ATP and crude extracts containing AmiR, it

was shown that AmiC had protein kinase activity and could phosphorylate a 66kDa protein present in the crude extracts. The nature of this protein remains unresolved, though the phosphorylation activity could be due to a relaxed specificity of AmiC which was present at high concentrations in the reactions. This would seem reasonable since this kinase activity did not appear to be affected by amide inducer. It was not possible to demonstrate AmiR phosphorylation by AmiC, since the background levels of phosphorylated protein in the cell were quite high and confused the analysis. However, it does seem likely that AmiC represses the antitermination activity of AmiR by phosphorylation. In the presence of amide inducer it is proposed that either the kinase activity of AmiC is curtailed, or that this is a bifunctional protein, similar to NR_{II} in *E.coli* and has phosphatase activity.

By this model AmiC and AmiR would form a sensory pair of proteins, with AmiC as the sensory protein, detecting the absence/presence of amide inducers and subsequently transferring this information to the transcriptional activator AmiR by a phosphorylation/(dephosphorylation) reaction. A large family of sensory pairs have now been identified in bacteria which utilise a common set of enzymatic activities for example NR_I/NR_{II}, EnvZ/OmpR and CheA/CheY (Stock *et al,* 1990) The sensory protein uses ATP to phosphorylate itself at a conserved histidine residue. The phosphoryl group is subsequently transferred to an aspartic side chain within a conserved domain of the regulatory protein (Stock *et al,* 1990). Similarly, BglF is phosphorylated by the cytoplasmic protein Hpr at His-547. This phosphate is subsequently transferred to His-306 within the same protein (Bramley and Kornberg, 1987b). His-306 can subsequently transfer the phosphate group to an un-identified site in BglG to inactivate it, in the absence of an inducer.

The phosphorylation experiments described in Chapter four suggested that AmiC was capable of transferring phosphate directly from ATP to another protein, and there was no evidence for the intermediate phosphorylation of

AmiC by another protein or self phosphorylation. This would suggest that AmiC does not fall into the class of histidine protein kinases (HPK) described above. In support of this argument, AmiC does not have any of the conserved residues, surrounding phosphorylated histidines found in other HPK's (Stock *et al,* 1990).

Conventional eukaryotic protein kinases contain a common catalytic domain which includes an N-terminal ATP binding site, a centrally located aspartic acid and a C-terminal protein substrate-binding region. The ATP-binding site is characterised by the Walker motif described in Section 6.4.2, whilst the central aspartic acid is responsible for base catalysed transfer of the phosphate to the protein substrate (for reviews see Kemp and Pearson, 1990; Taylor, 1989). AmiC does not have any sequences with homology to the consensus ATP binding site sequence. Consequently, AmiC might represent a novel form of protein kinase, which lacks an obvious ATP binding motif and yet is capable of direct phosphate transfer from ATP to a protein.

ii) Transcriptional regulation of the whole operon

The DNA sequencing described in Chapter 6 identified two additional open reading frames in the amidase operon, *amiY* and *amiX.* Thus giving the complete gene order for the operon, *amiEYCRX.* This analysis also revealed a potential transcription terminator sequence downstream of *amiE.*

Using primer extension analysis, the *amiE* transcription start was mapped and this confirmed that the consensus *E.coli* promoter previously identified (Drew and Lowe, 1989) was used in *E.coli* and *P.aeruginosa.* Transcription from this promoter was found to be constitutive, which is consistent with an antitermination model for regulated *amiE* expression. This analysis also showed a large difference in the level of transcription from the *amiE* promoter in *P.aeruginosa* compared with *E.coli.* This could be due to additional transcription factors in *P.aeruginosa* promoting transcription, such as a CAP analogue, which would recognise a *Pseudomonas* specific DNA sequence. Consistent with this hypothesis is the finding that in *P.aeruginosa,* amidase expres-

sion is sensitive to catabolite repression (Smyth and Clarke, 1975a).

The Northern analysis indicated that with the wild type chromosomal genes, in a non-induced state there was no detectable transcription of *amiE*. Similarly, there was no detectable transcription of *amiCRX*. In the induced state, a 1.3kb transcript encoding *amiE* was detected. The size of this transcript was in agreement with the mapped 5' end of the *amiE* transcript and the identification of a terminator, immediately downstream of *amiE*. The Northern analysis also showed that induced transcripts reading from the *amiE* promoter had the potential to read through the terminator downstream of *amiE* and continue through the entire operon to produce a 5.0kb transcript. The full length transcripts appeared to be particularly unstable and consistently hybridised with probes to produce a smear. This meant that accurate sizing of the undegraded transcript was not possible. Therefore the estimated size of 5.0kb for a full length transcript could have an error margin of up to 300 bases.

The search for a potential termination site 3' of *amiX*, for full length transcripts, has not identified any inverted repeats in the DNA sequence which might represent a transcriptional terminator. However, from the known start of transcription and the approximate size of the full length transcript, it is predicted that the termination site lies between the 3' end of *amiX* and the *SalI* target at position 5400.

S1 nuclease analysis was used to map the 3' end of the *amiE* transcript. These experiments confirmed that the axis of dyad symmetry 3' of the *amiE* gene was a site for transcription termination and the termination points were identified. It also showed that approximately 40% of induced transcripts proceeded through this terminator structure and thus had the potential to produce a polycistronic transcript encoding all the amidase genes as suggested by the Northern analysis. In this way, under inducing conditions, a gradient of transcripts is produced with 100% of transcripts encoding the *amiE* gene alone and 40% of transcripts encoding *amiEYCRX* (there is an additional complexity

to the gradient proposed which will be discussed later). By this model the induced levels of *amiR* expression would ensure maximal antitermination at the 5' *amiE* terminator and consequently maximal expression of the whole operon. However, this would not be a positive feedback loop, since *amiC* expression would be increased coordinately with *amiR* expression, thus ensuring sufficient AmiC was available to switch off the system when the inducer becomes limiting.

This model asks two questions:- i) How does the initial induction take place? This question is raised since with the chromosomal wild type genes, there was no detectable expression of *amiC* and *amiR* under non-inducing conditions and the model is reliant on AmiR to drive expression of itself from the *amiE* promoter. ii) What role do the *ntrA*-dependent promoters upstream of *amiC* play in regulation? The results from Chapter six, suggested they provide an additional control point for read through transcripts originating from the *amiE* promoter. These questions are dealt with below.

How does the initial induction of amidase expression occur?

Previous work (*in vivo)* had established there was promoter activity within an 800bp *Kpn*I(2400)-*Cla*I(3250) fragment from PAC433 upstream of *amiR* (Cousens *et al,* 1987). The analysis described in Chapter 3 further localised this promoter within a 658bp *Xho*I fragment from positions 2400-3050. Using the *amiE* promoter probe vector this promoter activity was shown to be weak and constitutive (pSW12). The DNA sequencing of *amiC* revealed two promoter elements with homology to the consensus *ntrA* dependent promoter sequences. However, primer extension studies provided no evidence for 5' ends of transcripts originating from these promoter elements. The failure of the primer extension analysis to identify the weak promoter upstream of *amiC* was largely attributed to read through transcripts from the *amiE* promoter which confused the analysis.

The *in vivo* demonstration of a weak constitutive promoter upstream of *amiC* provides an explanation for how the induction process occurs. In the

absence of inducer, this promoter would allow a basal level of AmiC and AmiR to be produced constitutively and AmiC would phosphorylate AmiR to repress its antitermination activity. In the presence of inducer, AmiC inactivation of AmiR would cease, allowing AmiR to cause antitermination at the *amiE* 5' terminator, which in turn would lead to increased expression of the entire operon as described above. Since the promoter activity immediately upstream of *amiC* is very weak, this would explain why it was not readily detectable by Northern analysis. This model is consistent with the model for the induction process in the *bgl* operon in which two weak constitutive promoters have been identified which ensure a basal level of expression of BglG the antiterminator, and BglF the repressor (Schnetz and Rak, 1988).

What is the role of the *ntrA* dependent promoters upstream of *amiC*

In Chapter six, it was proposed that σ^{54}-holoenzyme could form a stable closed complex at either of the two *ntrA* dependent promoter elements upstream of *amiC*. The presence of a stable closed complex was thought to provide a partial block for transcripts reading from the *amiE* promoter. In this way the level of *amiC* and *amiR* expression would be reduced, leading to reduced amidase activity. The evidence for this proposal was that deletion of these promoter elements gave higher levels of amidase activity, which would occur because transcription from the *amiE* promoter was proceeding unhindered. This would imply that the weaker downstream promoter was normally dominant, and yet there were no identifiable inverted repeats within the 658bp *Xho*I fragment which could terminate transcription from the *amiE* promoter. In addition amidase expression in an *ntrA*$^-$ host was increased up to 10 times in *E.coli* and up to 30 times in *P.aeruginosa* suggesting that it was not due to the lack of expression of an unlinked repressor gene transcribed from an *ntrA* dependent promoter.

Deletion of the promoter elements or the absence of NtrA are non-physiological events since *ntrA* is normally expressed constitutively. To address the normal role of these promoter elements, two points have to be examined.

Firstly, it has been shown that NtrC can cause increased expression of amidase and this phenomenon is dependent on the *ntrA* dependent promoters. This activation does not appear to result in the appearance of a major new transcript originating from either of these promoter elements (Section 6.9). This suggests that although NtrC might cause a small amount of transcription from these promoters, the major reason for increased amidase expression, results from transcription from the *amiE* promoter proceeding through these promoter elements unhindered by a closed complex. NtrC might mediate this effect by either allowing open complex formation and transcriptional initiation at a low rate or secondly destablising the closed complex structure.

The second point that has to be addressed is whether these results are an experimental artefact, or whether there is an activator protein in *P.aeruginosa* which acts at these promoters. If there is an activator in *P.aeruginosa* then it would not necessarily have to cause a large increase in expression from these promoters to cause a significant increase in amidase expression, as has been demonstrated with NtrC. Since the hydrolysis of amides by amidase leads to the production of ammonia, the most likely candidate for an activator in *P.aeruginosa* would be a component of the general nitrogen control system, such as an NtrC homologue.

Very little work has been done on the general nitrogen control system in *P.aeruginosa* though it is known that synthesis of several enzymes is increased in response to nitrogen limitation for example histidase, urocanase , glutamine synthetase and urease (Potts and Clarke, 1976; Janssen *et al*, 1980). The physiology of this regulation is poorly understood although it has been shown that the presence of excess glutamine in growth media causes repression of glutamine synthetase, histidase and urease production and a concomitant increase in NADP-dependent glutamate dehydrogenase production (Janssen *et al*, 1981).

Genetic studies of nitrogen control in *Pseudomonas* are equally scarce, although two types of regulatory mutants have been identified, termed

gln-2020 and *gln-2022*. The *gln-2020* mutation results in poor activation of glutamine synthetase and urease under nitrogen limiting growth conditions and only partial repression of glutamate dehydrogenase (Janssen *et al*, 1982b). It was proposed this mutation might be in a gene homologous to *ntrB* or *ntrC* in *E.coli*. The second chromosomal mutation, *gln-2022* was identified as a class of revertants from the *gln-2020* mutation. These mutants showed similar levels of glutamine synthetase and urease production under nitrogen excess or limiting growth conditions. They also showed high adenylation of glutamine synthetase under all growth conditions. Since the enzyme P_{II} (see chapter one) is involved in both the regulated expression of genes subject to nitrogen control and the adenylation of glutamine synthetase, it was proposed the *gln-2020* mutation might be within such a protein (Janssen *et al*, 1982b). Interestingly both these mutations are closely linked to glutamine synthetase. Together these results suggest there could be considerable similarity between the nitrogen control systems in enteric bacteria and *P.aeruginosa*.

If we assume that there is a general nitrogen controlled transcriptional activator, then could this protein regulate amidase expression? There are two contradictory answers to this question. Firstly in PAC strains, it has been shown that there is no escape from catabolite repression under nitrogen limiting conditions and even under catabolite derepressing conditions, there is no activation of amidase expression under nitrogen limitation. However, under catabolite repressing growth conditions it is possible to increase expression of histidase and urocanase by nitrogen limitation (Potts and Clarke, 1976). This would suggest that a general nitrogen activation system is still functional in PAC strains.

The contradictory observation with regard to amidase production is that of Janssen *et al* (1982a). Using strains PAO1 and PAO2175 they showed a hierachy for amidase expression as follows: maximal amidase expression was seen when acetamide was used as the sole source of carbon and nitrogen, the addition of acetamide and succinate to the growth medium caused a 33% drop in

amidase expression, due to catabolite repression. The addition of acetamide/ succinate and ammonia caused a further 5 fold decrease in amidase expression. These results showed that amidase expression in these strains was increased in response to nitrogen limitation. Why PAC strains don't show amidase activation under nitrogen limiting conditions remains a mystery. An investigation of the nitrogen regulation of amidase in other wild type *P.aeruginosa* strains would be useful, since it would indicate whether the behaviour of PAC strains was atypical.

In conclusion, one cannot say categorically whether the *ntrA* dependent promoters upstream of *amiC* are ever used in *vivo* in conjunction with a transcriptional activator, to drive expression of *amiC* and *amiR*. What has been established is that σ^{54}-holoenzyme binding to these promoters appears to be able to down regulate transcription proceeding from upstream, and presumably the cell must have some mechanism for switching this down-regulation off. NtrC can provide a physiological way for switching off this negative regulation in *E.coli*, so it seems likely that *P.aeruginosa* has a protein which can also perform this function.

iii) The Function of AmiY.

What do we know about *amiY* ? There are four pieces of information about *amiY* which will form the basis for a proposal of *amiY* function:

1) It is the second gene in the operon, and is expressed in response to an amide inducer.

2) The DNA sequencing showed that it contains an ATP binding motif.

3) D3112 mutator phage insertions which map in the *amiY* gene, give rise to strains which grow very poorly on acetamide plates (Rehmat and Shapiro, 1983).

4) A 400bp deletion within the *amiY* coding sequence does not appear to affect the high inducible expression seen from PAC452 pSW101 (R.Drew and S.Wachira, personal communication).

Since AmiY does not appear to be required for the induction of amidase expression and yet is produced in response to amides, one possible function

for AmiY could be as a permease responsible for the high level uptake of amides. To justify this proposal, the evidence for and against such a function will be examined.

An extensive search for an amide permease was carried out by Farin (1976). This work resulted in the identification of a constitutive acetate uptake system in *P.aeruginosa*. However these experiments provided no conclusive evidence for an amide permease and it was proposed that the plasma membrane of *P.aeruginosa* did not represent a permeability barrier to amides.

The ability of acetamide to enter both *P.aeruginosa* and *E.coli* by diffu sion is demonstrated by the nature of the transferase assay. In this assay acetamide (+hydroxylamine) is used as a substrate with **whole cell** suspensions and over a ten minute period, amidase activity is readily detected by the formation of a coloured complex with ferric chloride. This assay is completely AmiY independent since a plasmid with a 400bp deletion within the AmiY gene gives high amidase activities in the transferase assay (see above). Similarly the amidase activity from cell free extracts does not appear to be significantly higher than whole cell suspensions. However in these assays, saturating amounts of acetamide (0.1M) are added, therefore the diffusion of acetamide is not likely to be rate limiting.

Since there is no direct evidence that amiY is a permease, it can only be inferred from the data available. The following observations would be consistent with this proposal.

The D3112 mutator phage insertions in *amiY* produced strains which grew very poorly on acetamide plates (sole source of carbon and nitrogen) (Rehmat and Shapiro, 1983). This suggests that passive acetamide diffusion across the plasma membrane did not provide enough acetamide for growth. The wild type strain used for these studies with a functional *amiY* gene was capable of normal growth on acetamide plates.

Further evidence that amide permeation can be limiting under certain

conditions comes from the experiments with pSW41 in amidase constitutive strains of *P.aeruginosa*. With this transcomplementation system it was impossible to relieve AmiC repression of amidase synthesis, using lactamide. If we assume that the initial induction process relies on passive diffusion of an amide across the cell membrane (since high levels of AmiY would only be produced once the system was induced) then this would explain the above phenomenon. AmiC in this system is the major intracellular protein and consequently would require a large amount of amide to relieve its repression of AmiR. Passive diffusion clearly does not provide this level of amide required and consequently the system is never switched on (therefore no AmiY).

The preliminary observation that a 400bp deletion within the *amiY* coding sequence does not affect the inducibility of amidase expression is also consistent with the idea that AmiY is a permease. Since, by this model AmiY would not be required for induction, as the passive diffusion of amides across the cell membrane would be sufficient to inactivate the low levels of AmiC produced under non-inducing conditions.

In Gram-negative bacteria the main permeability barrier is the plasma membrane. There are four main systems by which nutrients are transported across the plasma membrane. With each system there is a requirement for an integral membrane protein, which facilitates the transport of molecules across the plasma membrane. Three of the systems have additional proteins which can be either cytoplasmically or periplasmically located. The cytoplasmic proteins are generally involved in energy transduction, whereas the periplasmic proteins usually bind the molecule to be transported There is only one class of transport system where a single, integral membrane protein is responsible for the whole process and this is typified by the proline transport system in enteric bacteria (Furlong, 1987).

If AmiY was a membrane bound protein then it would be predicted to contain hydrophobic transmembrane domains. Consequently a hydropathy plot was carried using the deduced amino acid sequence for AmiY by the method

of Kyte and Doolittle (1982). The results of this analysis are shown in Figure 7.2. This analysis indicates the AmiY sequence does not contain any hydrophobic regions which might represent transmembrane domains (Helen Stirk, personal communication). In fact the protein appears to be substantially hydrophillic suggesting it does not reside in the plasma membrane. Similarly the N-terminal sequence of AmiY does not have the charateristics of a signal sequence (Inouye and Halegoua, 1980; Oliver, 1987) which would allow transport into the periplasmic space. Together these findings would suggest that AmiY is normally located in the cytoplasm.

If AmiY is involved in the high level uptake of amides there would be a requirement for an additional transmembrane protein, which would work in conjunction with the cytoplasmic AmiY to allow uptake of amides. So far the *amiX* gene has not been taken into consideration and no role has been proposed for this protein. As with AmiY, AmiX is also produced in response to amide inducer. Could AmiX be a transmembrane protein which would work in conjunction with AmiY to allow amide uptake?

A hydropathy plot was carried out with the AmiX deduced sequence and the results are shown in Figure 7.3. This protein shows the classical pattern of strongly hydrophobic regions, characteristic of transmembrane helices. This analysis shows the presence of six substantially hydrophobic stretches of 18-22 residues, interspersed by short segments of 4-8 residues of charged, and conformationally flexible amino acids (Gly Ser, Pro). The length of the hydrophobic regions is strongly suggestive of transmembrane helices (*c.f.* bacterial rhodopsin). Taken together these observations suggest that AmiX would be an integral membrane protein.

Recently two related families of transport proteins have been identified in a wide range of organisms, concerned with the transport of many different compounds (for review see Blight and Holland, 1990). The first family is typified by HlyB which is involved in the secretion of haemolysin (HlyA) in *E.coli*. HlyB forms a membrane bound translocation complex with HlyD which allows

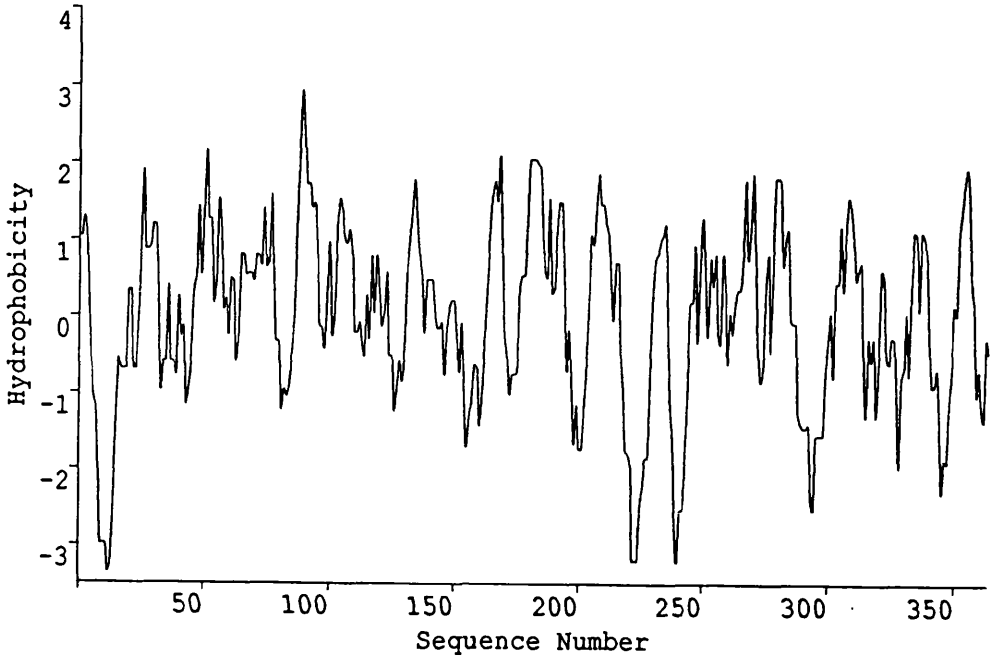


Figure 7.2. Hydropathy plot for the deduced AmiY sequence. The plot was prepared by the method of Kyte and Doolittle (1982) using a window size of 19 amino acids. Values greater than zero indicate hydrophobic regions and values less than zero, hydrophillic regions.

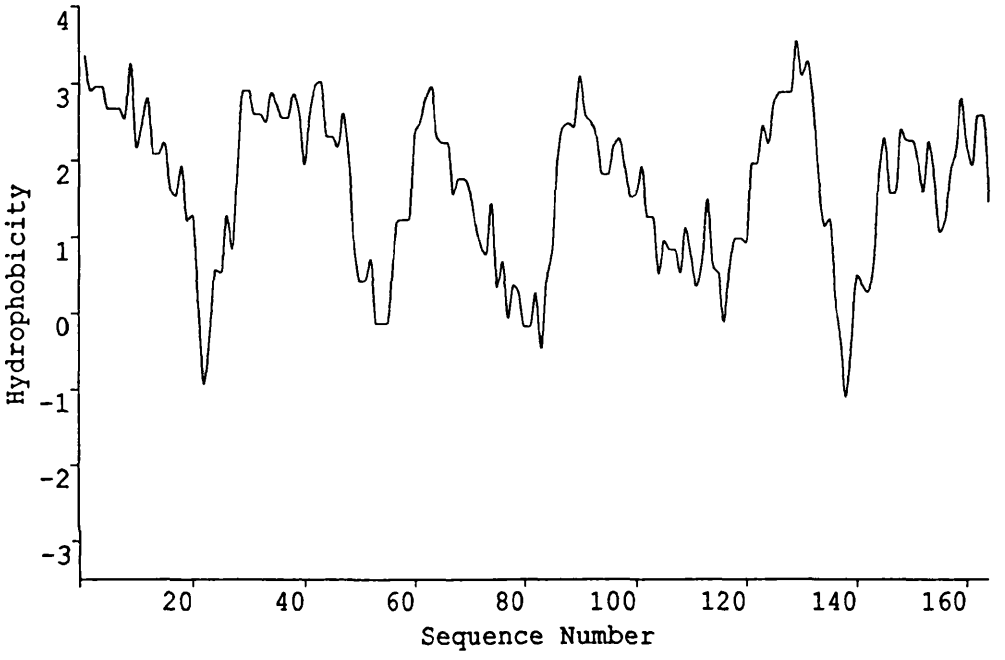


Figure 7.3. Hydropathy plot for the deduced AmiX sequence. The plot was prepared by the method of Kyte and Doolittle (1982) using a window size of 19 amino acids. Values greater than zero indicate hydrophobic regions and values less than zero, hydrophillic regions.

transport of the HlyA molecule directly to the medium without a periplasmic intermediate (Koronakis *et al*, 1989). The HlyB family of proteins includes; LktB of *Pasteurella haemolytica*, involved in Leukotoxin B secretion (Strathdee and Lo, 1989); the mammalian multi-drug resistance P-glycoprotein (Gerlach *et al*, 1986); the cystic fibrosis gene (Riordan *et al*, 1989).

This family of proteins have a similar structure, typified by HlyB, which includes a short hydrophillic N-terminal region which resides in the cytoplasm followed by a hydrophobic region which resides in the plasma membrane and forms eight transmembrane domains followed by a large C-terminal domain which contains the conserved part A and part B of a nucleotide binding motif (Blight and Holland, 1990). It is proposed that with HlyB the translocation of haemolysin via the N-terminal half of the protein, is coupled with the hydrolysis of ATP by the C-terminal region.

The second family of proteins which is related to the HlyB family are the HisP family of proteins. This family of proteins consists of cytoplasmic proteins (HisP) which couple energy to the uptake of small substrates through cytoplasmic membrane permeases in Gram-negative bacteria. The cytoplasmic proteins include MalK, HisP and OppD involved in the import across the cytoplasmic membrane of maltose, histidine and oligopepetides (Ames, 1985). These proteins are hydrophillic, lack signal sequences and, despite being tightly associated with the cytoplasmic membrane, are probably better described as peripheral membrane proteins. These proteins share extensive homology with the C-terminal domain of HlyB, around the predicted ATP-binding folds of this protein (Higgins *et al*, 1986) and consequently are proposed to be energy couplers in the transport system, deriving energy from the hydrolysis of ATP.

The uptake of histidine and maltose in *E.coli* involves four main proteins: With each system there are the hydrophillic ATPases described above. There are two integral cytoplasmic membrane proteins (MalG,MalF/HisM,HisQ) and a third binding protein located in the periplasm (HisJ/MalE) (Rosen, 1987).

It has been shown that the maltose binding protein MalE is not absolutely required for uptake of maltose (Shuman, 1982) and its main role is thought to be to bind maltose and present it to the MalF and MalG complex in the cytoplasmic membrane, thus ensuring efficient uptake of maltose.

The integral membrane protein, MalF, is proposed to have six-eight membrane spanning domains and MalG has been shown to comprise of six membrane spanning domains (Dassa and Hofnung, 1985). To summarise, the essential features of these two families are 1. The HlyB family are single proteins with a hydrophobic N-terminal half and a C-terminal domain which uses the energy derived from ATP hydrolysis to drive the excretion of products across the plasma membrane 2. The HisP family use a team of four proteins with a peripheral membrane protein which potentially hydrolyses ATP and is the energy transducer. Two integral membrane proteins, comprising of 6-8 transmembrane domains and a periplasmic binding protein.

It is proposed that AmiX and AmiY might form a two component transport system for aliphatic amides with similarity to the two families of transport proteins described above. The six transmembrane domains of AmiX suggest this is an integral membrane protein with a similar membrane topology to MalG. This protein would associate at the interface between the plasma membrane and the cytoplasm with AmiY which contains an ATP binding motif. In this model AmiY would be the energy transducing protein, utilisating the energy derived from ATP hydrolysis, and would be analogous to HisP. An alignment of the AmiY sequence with some HisP family members is shown in Figure 7.4.

A model for amidase regulation

Based on the data presented in this thesis the following model is proposed for amidase regulation. Some parts of the model have been proven whilst others remain hypothetical and require further experimentation The model is illustrated in Figure 7.5.

In the absence of an amide inducer there is low constitutive expression

```
MPFLSDMLDQSRRQQDEEQALARENLAEASLLQAHLSHRSALHSRFRFDPAAVMDCLRAEVLGQEPALQAVEDML
                                                    MMSENKLHVIDLHKRYGGHEVL
                                               MSLSETATQAPQPANVLLEVNDLRVTFATPDGDV
                                                                          :

KVVRADIADPRRPLFSALFLGPTGVGKTEIVRALARALHGDAEGFCRVDMNTLSQEHYAAALTGAPPGYVGAKEG
KGV    SLQARAGDVISIIGSSGSGKSTFLRCINF LEKPSEGAIIV  NGQNINLVRDKDGQLKVADKNQLRL
TAVN DLNFTLRAGETLGIVGESGSGKSQSRLRLMGLLATNGRIGGSATFNGREILNLPERELNTRRAEQISMIF
  *        *:  .  ::* :* **:     :   *   ..    :  *      .         :

TTLLEQDKLDGSPGRPGIVLFDELEKASPEVVHALINVLDNGLLRVASGERTYHFRNTLVFMTSNLCAHEIQRYD
LRTRLTMVFQH       FNLWSHM TVLENVMEAPIQVL  GLSKHDARERALKYLAKVGID
QDPMTSLNP        YMRVGEQLMEVLMLHK          GMSKAEAFEESVRMLDAVKMP
         .        . :  .:  :               *: :  . * : ::   :

ERRQRLPW RLLPVGGERRRRDIDGMVRARLLKTFSPEFVNRLDSVVTFNWIERDVVARLVELEVQRLNRRLEKH
ERAQ  GKYPVHLSGGQQQRVSIARALAMEPDVLLFDEPTSALDPELVGEVLRI
EARKRMKMYPHEFSGGMRQRVMIAMALLCRPKLLIADEPTTALDVTVQAQIMTLLNELKREFNTAIIMITHDLGV
*.....      . ** ..*  *  .:  .:  .:  * :. **  :   .:

  RCRLEATPEVLAKIARAGFDRQFGARALRRSV RHHLEVPLAEHLLDHHQ  PGDGNCTIYLASLEHERVRFV
 MQQLAEEGKTMVVVTHEMGFARHVSSHVIFLHQGKIEEEGDPEQVF        GNPQSPRLQQFLKGSLK*
VAGICDKVLVMYAGRTMEYGKARDVFYQPVHPYSIGLLNAVPRLDSEGAEMLTIPGNPPNLL  RLPKGCPFQPR
      .     .    . *  *.   ..:                                *::       :

RR*

CPHAMEICNNAPPLEAFSPGRLRACFKPVEELL*
```

Figure 7.4. Alignment of the amiY sequence with HisP (*E.coli*) and OppD (*Salmonella typhimurium*). The entire sequence of each protein is shown. AmiY is the top sequence, HisP the middle sequence and OppD the bottom sequence. "*" indicates complete identity, ":" indicates highly conservative amino acid changes and "." indicates similar amino acid usage.

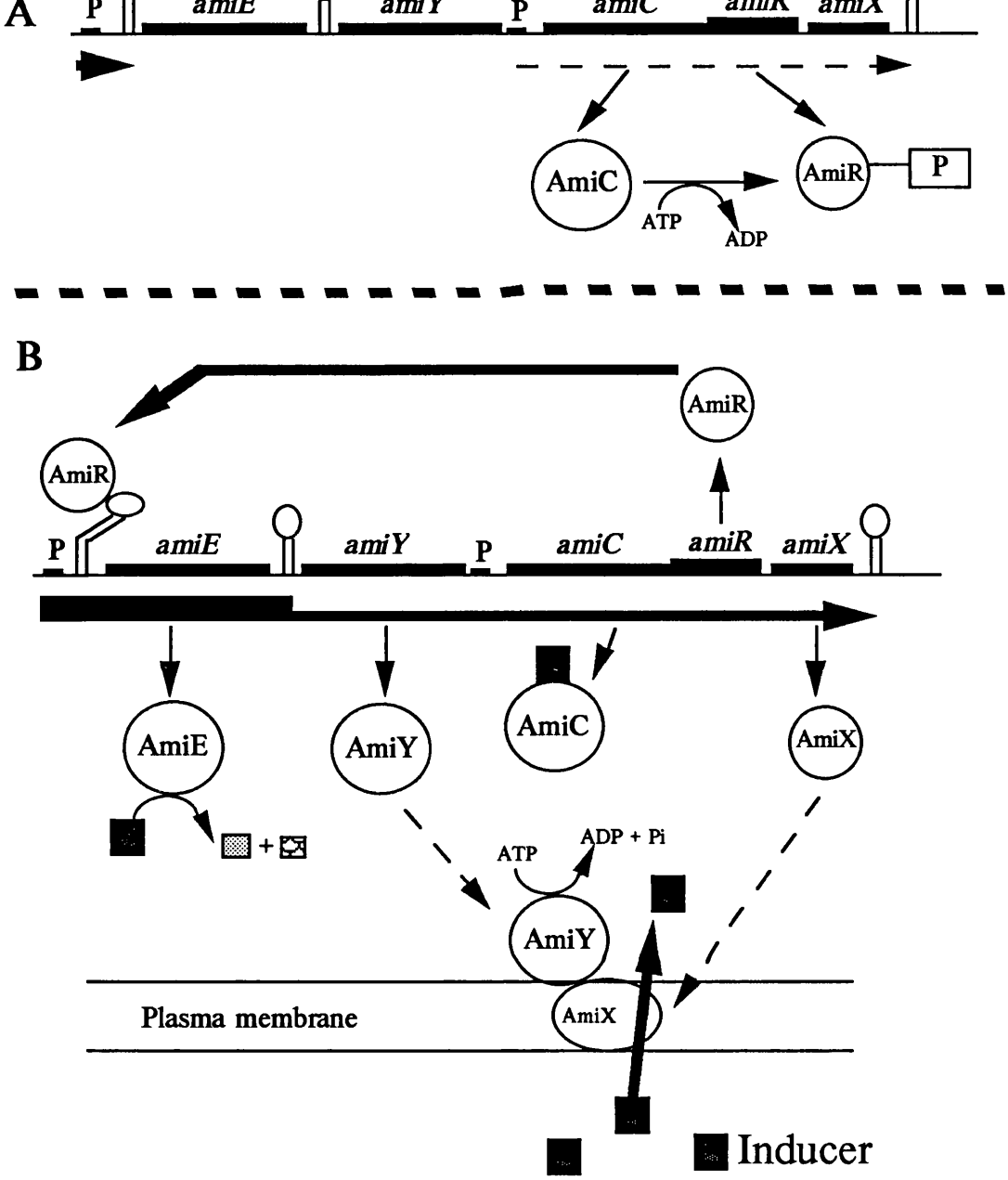


Figure 7.5. A model for the regulation of the amidase operon of *P.aeruginosa.* Part A shows a non-induced situation. The dashed arrow indicates a low level transcription of *amiC* and *amiR.* AmiC transfers phosphate from ATP to AmiR and the phosphorylated AmiR is inactive. Part B shows an induced situation. The shaded square represents the inducer. AmiX and AmiY allow high level uptake of inducer, which binds to AmiC preventing phosphorylation of AmiR. Functional AmiR causes transcription antitermination and allows maximal transcription of *amiE.* The terminator 3' of *amiE* allows 40% of transcripts reading from the *amiE* promoter to transcribe *amiYCRX* as indicated by the arrow beneath the genes. AmiE breaks down the inducer to provide both carbon and nitrogen sources for the cell. The initial induction occurs by passive diffusion of inducer across the plasma membrane (not shown). Stalks with loops indicate transcription terminators.

of *amiC* and *amiR*. AmiC phosphorylates AmiR and inactivates its antitermination activity. When an inducing amide is added to the growth medium, diffusion of the amide across the cell membrane takes place. The amide interaction with AmiC, curtails its protein kinase activity. At this stage, either AmiC dephosphorylates the small amount of AmiR present in the cell causing activation, or the next round of basal transcription of *amiC* and *amiR* results in the production of a small amount of functional AmiR.

AmiR then causes transcription antitermination at the *amiE* promoter region. This allows expression of *amiE*, and to a lesser extent, expression of *amiYCRX*. The increased levels of *amiR* expression ensure maximal antitermination and consequently maximal amidase expression, and high levels of AmiYCRX. AmiX and AmiY allow the high level transport of amides across the plasma membrane, ensuring efficient utilisation of external amides. This transport could be driven by ATP hydolysis. When the amide inducer becomes limiting, AmiC responds by phosphorylating AmiR, switching the system back to the non-induced state described above.

Two further refinements to this model are required to take into account catabolite repression of amidase synthesis and nitrogen regulation in PAO1. Nitrogen activation could occur by relief of the damping effect caused by σ^{54}-holoenzyme closed complex bound to the two *ntrA* dependent promoters upstream of *amiC*, possibly mediated by an *ntrC* homologue. Catabolite repression could occur at the *amiE* promoter since under catabolite derepressing conditions this constitutive promoter is much more active in *P.aeruginosa* than in *E.coli*, suggesting a positive regulation is occurring at this promoter in *P. aeruginosa*.

In conclusion, it would appear that amidase regulation in *P.aeruginosa* is very tightly regulated by an elegant mechanism which ensures the rapid switch on/off of amidase synthesis in response to alipathic amides in the environment.

```
      1                   20                  40
      AAGCTTCCGTCGGAATGATGGCATGCATGCTATCTCAGGCTCGCACCATGTGCTTT

  60                  80                  100
CGCGATCGCGCCGATTACATAACGTTACACGAACCTTGACAGCCCCTTCCGACGGGGCTTAT

                    140                 160                 180
AAGTGGCGCCATCAGGTCATGCGCATCAGCGTCGATGTCGCGGGACCGAACCTAACGCATAC

                    200                 220                 240
GCACAGAGCAAATGGGCTCTCCCGGGGTTACCCGGGAGGGCCTTTTTTCGTCCCGAAAAAAT

                260                 280
AACAACAAGAGGTGATACCC ATG CGT CAC GGC GAT ATT TCC AGC AGC AAC GAC ACC
                     Met Arg His Gly Asp Ile Ser Ser Ser Asn Asp Thr

                    320                 340
GTC GGA GTG GCC GTG GTC AAC TAC AAG ATG CCG CGC CTG CAC ACC GCG GCG GAG
Val Gly Val Ala Val Val Asn Tyr Lys Met Pro Arg Leu His Thr Ala Ala Glu

        360                 380                 400
GTC CTG GAC AAC GCC CGG AAG ATC GCC GAC ATG ATC GTC GGC ATG AAG CAG GGC
Val Leu Asp Asn Ala Arg Lys Ile Ala Asp Met Ile Val Gly Met Lys Gln Gly

                420                 440                 460
CTG CCC GGC ATG GAC CTG GTG GTG TTC CCG GAG TAC AGC CTG CAG GGC ATC ATG
Leu Pro Gly Met Asp Leu Val Val Phe Pro Glu Tyr Ser Leu Gln Gly Ile Met

                480                 500
TAC GAT CCG GCG GAG ATG ATG GAA ACC GCG GTG GCG ATC CCC GGC GAG GAA ACC
Tyr Asp Pro Ala Glu Met Met Glu Thr Ala Val Ala Ile Pro Gly Glu Glu Thr

    520                 540                 560
GAG ATA TTC TCC CGC GCC TGC CGC AAG GCC AAC GTC TGG GGC GTA TTC TCC CTC
Glu Ile Phe Ser Arg Ala Cys Arg Lys Ala Asn Val Trp Gly Val Phe Ser Leu

            580                 600                 620
ACC GGC GAA CGG CAC GAG GAG CAT CCG CGC AAG GCG CCG TAC AAC ACC CTG GTG
Thr Gly Glu Arg His Glu Glu His Pro Arg Lys Ala Pro Tyr Asn Thr Leu Val

                640                 660
CTG ATC GAC AAC AAC GGC GAG ATC GTC CAG AAG TAC CGC AAG ATC ATT CCC TGG
Leu Ile Asp Asn Asn Gly Glu Ile Val Gln Lys Tyr Arg Lys Ile Ile Pro Trp

  680                 700                 720
TGC CCC ATC GAG GGC TGG TAT CCC GGT GGC CAG ACC TAC GTC AGC GAA GGG CCG
Cys Pro Ile Glu Gly Trp Tyr Pro Gly Gly Gln Thr Tyr Val Ser Glu Gly Pro

        740                 760                 780
AAG GGC ATG AAG ATC AGC CTG ATC ATC TGC GAC GAC GGC AAT TAC CCG GAG ATC
Lys Gly Met Lys Ile Ser Leu Ile Ile Cys Asp Asp Gly Asn Tyr Pro Glu Ile

                800                 820
TGG CGC GAC TGC GCG ATG AAG GGC GCC GAG CTG ATC GTG CGC TGC CAG GGC TAC
Trp Arg Asp Cys Ala Met Lys Gly Ala Glu Leu Ile Val Arg Cys Gln Gly Tyr

                860                 880
ATG TAC CCG GCC AAG GAC CAG CAG GTG ATG ATG GCC AAG GCC ATG GCC TGG GCC
Met Tyr Pro Ala Lys Asp Gln Gln Val Met Met Ala Lys Ala Met Ala Trp Ala

      900                 920                 940
AAC AAC TGC TAT GTG GCG GTG GCC AAC GCG GCC GGC TTC GAC GGT GTC TAT TCC
Asn Asn Cys Tyr Val Ala Val Ala Asn Ala Ala Gly Phe Asp Gly Val Tyr Ser

            960                 980                1000
TAC TTC GGC CAC TCG GCG ATC ATC GGC TTC GAC GGC CGT ACC CTC GGT GAG TGC
Tyr Phe Gly His Ser Ala Ile Ile Gly Phe Asp Gly Arg Thr Leu Gly Glu Cys

                1020                1040
GGC GAG GAG GAA ATG GGT ATC CAG TAC GCC CAG CTG TCC CTT TCG CAG ATC CGC
Gly Glu Glu Glu Met Gly Ile Gln Tyr Ala Gln Leu Ser Leu Ser Gln Ile Arg

   1060                1080                1100
GAT GCG CGC GCC AAC GAT CAG TCG CAG AAC CAC CTG TTC AAG ATC CTC CAC CGC
Asp Ala Arg Ala Asn Asp Gln Ser Gln Asn His Leu Phe Lys Ile Leu His Arg

            1120                1140                1160
GGC TAC AGC GGC TTG CAG GCG TCC GGC GAC GGC GAC CGG GGC CTG GCG GAG TGT
Gly Tyr Ser Gly Leu Gln Ala Ser Gly Asp Gly Asp Arg Gly Leu Ala Glu Cys

                1180                1200
CCG TTC GAG TTC TAC CGC ACC TGG GTC ACC GAC GCC GAG AAG GCG CGC GAC AAT
Pro Phe Glu Phe Tyr Arg Thr Trp Val Thr Asp Ala Glu Lys Ala Arg Asp Asn
```

```
1220                        1240                        1260
GTC GAG CGA CTG ACC CGC TCG ACC ACC GGC GTG GCG CAA TGC CCG GTC GGC CGG
Val Glu Arg Leu Thr Arg Ser Thr Thr Gly Val Ala Gln Cys Pro Val Gly Arg

            1280                        1300                        1320
CTG CCC TAT GAG GGA CTG GAG AAG GAA GCC TGA CGGCAGACGCCGCCAGCCCCGG
Leu Pro Tyr Glu Gly Leu Glu Lys Glu Ala ---                      <<<<

            1340                        1360                        1380
CGTGCCGTCGTGCGGCACGCCGTCTCCGCCATTTCCCCCCTGTGGCAGAAGGAGTTTCATCC
<<<<<<<<<<  >>>>>>>>>>>>>>>>

                1400                        1420                        1440
ATG CCT TTT CTG AGC GAC ATG CTC GAC CAG TCC CGC CGG CAG CAG GAC GAG GAA
Met Pro Phe Leu Ser Asp Met Leu Asp Gln Ser Arg Arg Gln Gln Asp Glu Glu

                        1460                        1480
CAG GCC CTG GCG CGG GAG AAT CTT GCC GAG GCA AGT CTG CTC CAG GCC CAC CTG
Gln Ala Leu Ala Arg Glu Asn Leu Ala Glu Ala Ser Leu Leu Gln Ala His Leu

    1500                        1520                        1540
AGT CAC CGC AGC GCC CTG CAC AGC CGT TTC CGT TTC GAC CCG GCG GCG GTG ATG
Ser His Arg Ser Ala Leu His Ser Arg Phe Arg Phe Asp Pro Ala Ala Val Met

            1560                        1580                        1600
GAC TGC CTG CGC GCC GAG GTG CTC GGC CAG GAA CCG GCG CTA CAG GCC GTC GAG
Asp Cys Leu Arg Ala Glu Val Leu Gly Gln Glu Pro Ala Leu Gln Ala Val Glu

                        1620                        1640
GAC ATG CTC AAG GTG GTT CGC GCG GAC ATC GCC GAC CCG CGC CGT CCG CTG TTC
Asp Met Leu Lys Val Val Arg Ala Asp Ile Ala Asp Pro Arg Arg Pro Leu Phe

 1660                        1680                        1700
AGC GCG CTG TTC CTC GGC CCC ACC GGA GTC GGC AAG ACC GAG ATC GTG CGC GCC
Ser Ala Leu Phe Leu Gly Pro Thr Gly Val Gly Lys Thr Glu Ile Val Arg Ala

            1720                        1740                        1760
CTG GCC AGG GCC CTG CAC GGC GAC GCC GAG GGG TTC TGC CGG GTG GAC ATG AAC
Leu Ala Arg Ala Leu His Gly Asp Ala Glu Gly Phe Cys Arg Val Asp Met Asn

                        1780                        1800
ACC CTG TCC CAG GAG CAC TAT GCC GCC GCC CTC ACC GGT GCG CCG CCG GGC TAC
Thr Leu Ser Gln Glu His Tyr Ala Ala Ala Leu Thr Gly Ala Pro Pro Gly Tyr

                        1840                        1860
GTC GGG GCG AAG GAG GGC ACC ACC CTG TTG GAG CAG GAC AAG CTG GAC GGC AGT
Val Gly Ala Lys Glu Gly Thr Thr Leu Leu Glu Gln Asp Lys Leu Asp Gly Ser

    1880                        1900                        1920
CCC GGG CGC CCC GGC ATC GTT CTC TTC GAC GAA CTG GAA AAG GCC AGC CCG GAA
Pro Gly Arg Pro Gly Ile Val Leu Phe Asp Glu Leu Glu Lys Ala Ser Pro Glu

                1940                        1960                        1980
GTG GTC CAT GCG TTG CTC AAC GTA CTC GAC AAC GGC CTG CTA CGG GTC GCT TCC
Val Val His Ala Leu Leu Asn Val Leu Asp Asn Gly Leu Leu Arg Val Ala Ser

                        2000                        2020
GGC GAA CGC ACC TAC CAT TTC CGC AAC ACC CTG GTG TTC ATG ACC AGC AAT CTC
Gly Glu Arg Thr Tyr His Phe Arg Asn Thr Leu Val Phe Met Thr Ser Asn Leu

    2040                        2060                        2080
TGC GCC CAT GAG ATC CAG CGC TAC GAC GAG CGT CGC CAG CGC CTG CCC TGG CGC
Cys Ala His Glu Ile Gln Arg Tyr Asp Glu Arg Arg Gln Arg Leu Pro Trp Arg

            2100                        2120                        2140
CTG CTG CCG GTC GGC GGC GAG CGC CGG CGG CGG GAC ATC GAC GGG ATG GTC CGG
Leu Leu Pro Val Gly Gly Glu Arg Arg Arg Arg Asp Ile Asp Gly Met Val Arg

                        2160                        2180
GCC CGG CTG CTG AAG ACC TTC TCG CCG GAG TTC GTC AAT CGT CTC GAT AGC GTG
Ala Arg Leu Leu Lys Thr Phe Ser Pro Glu Phe Val Asn Arg Leu Asp Ser Val

 2200                        2220                        2240
GTC ACC TTC AAC TGG ATC GAA CGC GAC GTC GTC GCG CGC CTG GTC GAG CTG GAG
Val Thr Phe Asn Trp Ile Glu Arg Asp Val Val Ala Arg Leu Val Glu Leu Glu

            2260                        2280                        2300
GTG CAG CGG CTC AAC CGG CGC CTG GAG AAG CAT CGC TGC CGC CTG GAG GCG ACC
Val Gln Arg Leu Asn Arg Arg Leu Glu Lys His Arg Cys Arg Leu Glu Ala Thr

                        2320                        2340
CCG GAG GTG CTG GCG AAG ATC GCC CGC GCC GGC TTC GAC CGG CAG TTC GGC GCC
Pro Glu Val Leu Ala Lys Ile Ala Arg Ala Gly Phe Asp Arg Gln Phe Gly Ala
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                                  2380                                2400 CTG GYA YRN NNN
CGT GCG TTG CGC CGC AGC GTC CGT CAT CAT CTC GAG GTA CCG CTG GCC GAG CAT
Arg Ala Leu Arg Arg Ser Val Arg His His Leu Glu Val Pro Leu Ala Glu His

TTG CA                                2440                          CTG GYA YRN
CTG CTC GAT CAC CAC CAG CCG GGC GAC GGG AAC TGC ACG ATC TAC CTG GCG AGC
Leu Leu Asp His His Gln Pro Gly Asp Gly Asn Cys Thr Ile Tyr Leu Ala Ser

NNN TTG CA    2480                          2500                          2520
CTG GAG CAC GAG CGG GTT CGC TTC GTA CGG CGC TGA GCGACAGTCACAGGAGAGGA
Leu Glu His Glu Arg Val Arg Phe Val Arg Arg ---

                      2540                          2560
AACGG ATG GGA TCG CAC CAG GAG CGG CCG CTG ATC GGC CTG CTG TTC TCC GAA
      Met Gly Ser His Gln Glu Arg Pro Leu Ile Gly Leu Leu Phe Ser Glu

  2580                          2600                          2620
ACC GGC GTC ACC GCC GAT ATC GAG CGC TCG CAC GCG TAT GGC GCA TTG CTC GCG
Thr Gly Val Thr Ala Asp Ile Glu Arg Ser His Ala Tyr Gly Ala Leu Leu Ala

          2640                          2660                          2680
GTC GAG CAA CTG AAC CGC GAG GGC GGC GTC GGC GGT CGC CCG ATC GAA ACG CTG
Val Glu Gln Leu Asn Arg Glu Gly Gly Val Gly Gly Arg Pro Ile Glu Thr Leu

                      2700                          2720
TCC CAG GAC CCC GGC GGC GAC CCG GAC CGC TAT CGG CTG TGC GCC GAG GAC TTC
Ser Gln Asp Pro Gly Gly Asp Pro Asp Arg Tyr Arg Leu Cys Ala Glu Asp Phe

                              2760                          2780
ATT CGC AAC CGG GGG GTA CGG TTC CTC GTG GGC TGC TAC ATG TCG CAC ACG CGC
Ile Arg Asn Arg Gly Val Arg Phe Leu Val Gly Cys Tyr Met Ser His Thr Arg

        2800                          2820                          2840
AAG GCG GTG ATG CCG GTG GTC GAG CGC GCC GAC GCG CTG CTC TGC TAC CCG ACC
Lys Ala Val Met Pro Val Val Glu Arg Ala Asp Ala Leu Leu Cys Tyr Pro Thr

                  2860                          2880
CCC TAC GAG GGC TTC GAG TAT TCG CCG AAC ATC GTC TAC GGC GGT CCG GCG CCG
Pro Tyr Glu Gly Phe Glu Tyr Ser Pro Asn Ile Val Tyr Gly Gly Pro Ala Pro

                          2920                          2940
AAC CAG AAC AGT GCG CCG CTG GCG GCG TAC CTG ATT CGC CAC TAC GGC GAG CGG
Asn Gln Asn Ser Ala Pro Leu Ala Ala Tyr Leu Ile Arg His Tyr Gly Glu Arg
    2960                          2980                          3000
GTG GTG TTC ATC GGC TCG GAC TAC ATC TAT CCG CGG GAA AGC AAC CAT GTG ATG
Val Val Phe Ile Gly Ser Asp Tyr Ile Tyr Pro Arg Glu Ser Asn His Val Met

              3020                          3040                          3060
CGC CAC CTG TAT CGC CAG CAC GGC GGC ACG GTG CTC GAG GAA ATC TAC ATT CCG
Arg His Leu Tyr Arg Gln His Gly Gly Thr Val Leu Glu Glu Ile Tyr Ile Pro

                      3080                          3100
CTG TAT CCC TCC GAC GAC GAC TTG CAG CGC GCC GTC GAG CGC ATC TAC CAG GCG
Leu Tyr Pro Ser Asp Asp Asp Leu Gln Arg Ala Val Glu Arg Ile Tyr Gln Ala

  3120                          3140                          3160
CGC GCC GAC GTG GTC TTC TCC ACC GTG GTG GGC ACC GGC ACC GCC GAG CTG TAT
Arg Ala Asp Val Val Phe Ser Thr Val Val Gly Thr Gly Thr Ala Glu Leu Tyr

          3180                          3200                          3220
CGC GCC ATC GCC CGT CGC TAC GGC GAC GGC AGG CGG CCG CCG ATC GCC AGC CTG
Arg Ala Ile Ala Arg Arg Tyr Gly Asp Gly Arg Arg Pro Pro Ile Ala Ser Leu

                      3240                          3260
ACC ACC AGC GAG GCG GAG GTG GCG AAG ATG GAG AGT GAC GTG GCA GAG GGG CAG
Thr Thr Ser Glu Ala Glu Val Ala Lys Met Glu Ser Asp Val Ala Glu Gly Gln

                              3300                          3320
GTG GTG GTC GCG CCT TAC TTC TCC AGC ATC GAT ACG CCC GCC AGC CGG GCC TTC
Val Val Val Ala Pro Tyr Phe Ser Ser Ile Asp Thr Pro Ala Ser Arg Ala Phe

        3340                          3360                          3380
GTC CAG GCC TGC CAT GGT TTC TTC CCG GAG AAC GCG ACC ATC ACC GCC TGG GCC
Val Gln Ala Cys His Gly Phe Phe Pro Glu Asn Ala Thr Ile Thr Ala Trp Ala

                  3400                          3420
GAG GCG GCC TAC TGG CAG ACC TTG TTG CTC GGC CGC GCC GCG CAG GCC GCA GGC
Glu Ala Ala Tyr Trp Gln Thr Leu Leu Leu Gly Arg Ala Ala Gln Ala Ala Gly

                          3460                          3480
AAC TGG CGG GTG GAA GAC GTG CAG CGG CAC CTG TAC GAC ATC GAC ATC GAC GCG
Asn Trp Arg Val Glu Asp Val Gln Arg His Leu Tyr Asp Ile Asp Ile Asp Ala
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    3500                        3520                        3540
CCA CAG GGG CCG GTC CGG GTG GAG CGC CAG AAC AAC CAC AGC CGC CTG TCT TCG
Pro Gln Gly Pro Val Arg Val Glu Arg Gln Asn Asn His Ser Arg Leu Ser Ser

            3560                        3580                        3600
CGC ATC GCG GAA ATC GAT GCG CGC GGC GTG TTC CAG GTC CGC TGG CAG TCG CCC
Arg Ile Ala Glu Ile Asp Ala Arg Gly Val Phe Gln Val Arg Trp Gln Ser Pro

                    3620                        3640
GAA CCG ATT CGC CCC GAC CCT TAT GTC GTC GTG CAT AAC CTC GAC GAC TGG TCC
Glu Pro Ile Arg Pro Asp Pro Tyr Val Val Val His Asn Leu Asp Asp Trp Ser

  3660                        3680                        3700
GCC AGC ATG GGC GGG GGA GCG CTC CCA TG AGC GCC ACC TCG CTG CTC GGC AGC
Ala Ser Met Gly Gly Gly Ala Leu Pro -- -
                                    Met Ser Ala Asn Ser Leu Leu Gly Ser

            3720                        3740                        3760
CTG CGC GAG TTG CAG GTG CTG GTC CTC AAC CCG CCG GGG GAG GTC AGC GAC GCC
Leu Arg Glu Leu Gln Val Leu Val Leu Asn Pro Pro Gly Glu Val Ser Asp Ala

                    3780                        3800
CTG GTC TTG CAG CTG ATC CGC ATC GGT TGT TCG GTG CGC CAG TGC TGG CCG CCG
Leu Val Leu Gln Leu Ile Arg Ile Gly Cys Ser Val Arg Gln Cys Trp Pro Pro

 3820                        3840                        3860
CCG GAA GCC TTC GAC GTG CCG GTG GAC GTG GTC TTC ACC AGC ATT TTC CAG AAT
Pro Glu Ala Phe Asp Val Pro Val Asp Val Val Phe Thr Ser Ile Phe Gln Asn

            3880                        3900                        3920
GGC CAC CAC GAC GAG ATC GCT GCG CTG CTC GCC GCC GGG ACT CCG CGC ACT ACC
Gly His His Asp Glu Ile Ala Ala Leu Leu Ala Ala Gly Thr Pro Arg Thr Thr

                    3940                        3960
CTG GTG GCG CTG GTG GAG TAC GAA AGC CCC GCG GTG CTC TCG CAG ATC ATC GAG
Leu Val Ala Leu Val Glu Tyr Glu Ser Pro Ala Val Leu Ser Gln Ile Ile Glu

                        4000                        4020
CTG GAG TGC CAC GGC GTG ATC ACC CAG CCG CTC GAT GCC CAC CGG GTG CTG CCT
Leu Glu Cys His Gly Val Ile Thr Gln Pro Leu Asp Ala His Arg Val Leu Pro

    4040                        4060                        4080
GTG CTG GTA TCG GCG CGG CGC ATC AGC GAG GAA ATG GCG AAG CTG AAG CAG AAG
Val Leu Val Ser Ala Arg Arg Ile Ser Glu GluMet Ala Lys Leu Lys Gln Lys

            4100                        4120                        4140
ACC GAG CAG CTC CAG GAC CGC ATC GCC GGC CAG GCC CGG ATC AAC CAG GCC AAG
Thr Glu Gln Leu Gln Asp Arg Ile Ala Gly Gln Ala Arg Ile Asn Gln Ala Lys

                    4160                        4180
GTG TTG CTG ATG CAG CGC CAT GGC TGG GAC GAG CGC GAG GCG CAC CAG CAC CTG
Val Leu Leu Met Gln Arg His Gly Trp Asp Glu Arg Glu Ala His Gln His Leu

   4200                        4220                        4240
TCG CGG GAA GCG ATG AAG CGG CGC GAG CCG ATC CTG AAG ATC GCT CAG GAG TTG
Ser Arg Glu Ala Met Lys Arg Arg Glu Pro Ile Leu Lys Ile Ala Gln Glu Leu

            4260                        4280                        4300
CTG GGA AAC GAG CCG TCC GCC TGA GCGATCCGGGCCGACCAGAACAATAACAAGAGGGG
Leu Gly Asn Glu Pro Ser Ala ---

            4320                        4340                        4360
TATCGTCATC ATG CTG GGA CTG GTT CTG CTG TAC GTT GGC GCG GTG CTG TTT CTC
               Met Leu Gly Leu Val Leu Leu Tyr Val Gly Ala Val Leu Phe Leu

                    4380                        4400
AAT GCC GTC TGG TTG CTG GGC AAG ATC AGC GGT CGG GAG GTG GCG GTG ATC AAC
Asn Ala Val Trp Leu Leu Gly Lys Ile Ser Gly Arg Glu Val Ala Val Ile Asn

 4420                        4440                        4460
TTC CTG GTC GGC GTG CTG AGC GCC TGC GTC GCG TTC TAC CTG ATC TTT TCC GCA
Phe Leu Val Gly Val Leu Ser Ala Cys Val Ala Phe Tyr Leu Ile Phe Ser Ala

            4480                        4500                        4520
GCA GCC GGG CAG GGC TCG CTG AAG GCC GGA GCG CTG ACC CTG CTA TTC GCT TTT
Ala Ala Gly Gln Gly Ser Leu Lys Ala Gly Ala Leu Thr Leu Leu Phe Ala Phe

                    4540                        4560
ACC TAT CTG TGG GTG GCC GCC AAC CAG TTC CTC GAG GTG GAC GGC AAG GGC CTC
Thr Tyr Leu Trp Val Ala Ala Asn Gln Phe Leu Glu Val Asp Gly Lys Gly Leu
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                                4600                                4620
GGC TGG TTC TGC CTG TTC GTC AGC CTC ACC GCC TGC ACC GTG GCG ATC GAG TCG
Gly Trp Phe Cys Leu Phe Val Ser Leu Thr Ala Cys Thr Val Ala Ile Glu Ser

      4640                              4660                              4680
TTC GCC GGC GCC AGT GGT CCG TTC GGC CTG TGG AAC GCG GTC AAC TGG ACA GTC
Phe Ala Gly Ala Ser Gly Pro Phe Gly Leu Trp Asn Ala Val Asn Trp Thr Val

               4700                              4720                              4740
TGG GCG TTG CTC TGG TTC TGT TTC TTC CTG CTG CTG GGG CTG TCC CGC GGC ATC
Trp Ala Leu Leu Trp Phe Cys Phe Phe Leu Leu Leu Gly Leu Ser Arg Gly Ile

                          4760                              4780
CAG AAG CCG GTG GCC TAC CTG ACC CTG GCC AGC GCC ATA TTC ACC GCC TGG TTG
Gln Lys Pro Val Ala Tyr Leu Thr Leu Ala Ser Ala Ile Phe Thr Ala Trp Leu

   4800                              4820                              4840
CCC GGC CTG CTG CTG CTC GGA CAG GTG CTC AAG GCA TAG CAGGAAGTCGGAAAGGG
Pro Gly Leu Leu Leu Leu Gly Gln Val Leu Lys Ala ---

       4860                              4880                              4900
ATGACGGCTTGCCGCCATCCCGTCCCTTCCGAACGCCTAGCCGAGCGGCCAGTTGATCACCA

     4920                              4940                              4960
CGACGGCGTCGTTGTAGTCGTTGTCGGTGCCGTCTTCAGAGCCGACCAGGGCGAAGTTCAGC

   4980                              5000                              5020
TCGTTGGTCAGGATTACCTGTGCCGAGACCAGATCCGAGGGGCGGCCGTTGACGCTGACCTG

5040                              5060                              5080
GACCTGTACCTTGCCACTGCTGCCGGAGTTGAGCACCTGGGTGCCGATGACGGCGTTATTGG

                          5120                              5140                              5160
TGCTTTGCCCGCTGAAGGTCGCGGCCGTGCTCGTTGTTGACCAGCACGTTCACCGTCTGGGT

                     5180                              5200                              5220
TCCGGACGAGTTGGCGAAGGCGGTGACGCCGGAACCTGGTTGTTGGCGGGAAGGGTGAACAC

                5240                              5260                              5280
TCCTTGTGGTTGCCATGGTGGTATCTCCACTGAATACCTGGCCCCTTCCTTTTCAGGCAGCC

           5300                              5320                              5340
GTCTGGCGCGCGGTATGGCGTGTCGGGAGAAATCCGCAGTCCTTGGCGGCAGGCGATGCGCA

      5360                              5380
GGCAGGAAGGACGCATCGTTCAGCCAATCTACGCCGTCGAC
```

Appendix I. Nucleotide sequence of the 5.4kb *Hin*dIII-*Sal*I fragment encoding the amidase genes of *P.aeruginosa*. Gene order is ***amiE, amiY, amiC, amiR***, *amiX*.

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