Complementation Analysis of the Aliphatic Amidase Genes of *Pseudomonas aeruginosa*

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A plasmid, pCL34, capable of autonomous replication in *Escherichia coli* and *Pseudomonas aeruginosa* has been constructed which carries the promoter and structural gene (*amiE*) for *P. aeruginosa* amidase, but not the regulator gene (*amiR*). Plasmid pCL34 has been mobilized from *E. coli* to *P. aeruginosa* using the broad host range plasmid RP4. Complementation studies were performed in *P. aeruginosa* strains carrying various amidase mutations. Measurements of amidase activity in the recipients under inducing, non-inducing and repressing conditions showed *trans*-complementation by the chromosomally located regulator gene product. These results confirmed the positive control model for amidase gene expression. Levels of amidase expression seen during these studies were approximately threefold higher than in the parental, amidase-positive strains.

INTRODUCTION

Studies on the control of gene expression in *Pseudomonas aeruginosa* have been hampered by the inability to perform classical complementation analysis with merodiploids. However, using the wide host range IncP-1 plasmid R68.45 (Haas & Holloway, 1976), R' plasmids from *P. aeruginosa* have been isolated by selection in *Escherichia coli* (Hedges & Jacob, 1977; Hedges *et al.*, 1977), from recombination deficient *P. aeruginosa* (Holloway, 1978) and *P. putida* (Morgan, 1982). Such R' plasmids have been found to be unstable on transfer to recombination proficient *P. aeruginosa* strains and not useful for complementation studies.

In *E. coli*, studies of the mechanism of control of gene expression by complementation analysis have been utilized in the examination of classic negatively controlled systems, such as the lactose operon (Jacob & Monod, 1961), of more complex positively controlled systems including D-serine deaminase (Bloom *et al.*, 1975; Carothers *et al.*, 1980), and of those with aspects of both positive and negative control, the arabinose operon (Englesberg *et al.*, 1965, 1969). In these cases the dominance relationships between regulator gene products in the merodiploid state have provided one line of evidence towards defining the mechanisms of regulation.

Expression of the aliphatic amidase gene (*amiE*) of *P. aeruginosa* (Kelly & Clarke, 1962) is under the control of the closely linked regulator gene product (Brammar *et al.*, 1967). Farin & Clarke (1978) concluded that the *amiR* gene product exerted positive control on the expression of the *amiE* gene. Amidase synthesis is also subject to catabolite repression by succinate (Smyth & Clarke, 1975*a*). Mutants showing resistance to catabolite repression fall into at least two classes: (i) those closely linked to the amidase genes and considered to be up-promoter mutants and (ii) those unlinked to the amidase genes (Smyth & Clarke, 1975*a, b*). Hybrid λ *ami* recombinant bacteriophages constructed *in vitro* have been described previously (Drew *et al.*, 1980). The amidase structural gene and its promoter from an up-promoter mutant have been subcloned into plasmid pBR322 and the structural gene located (Clarke *et al.*, 1981) and partially sequenced (W. J. Brammar, personal communication).

I have deleted non-essential *P. aeruginosa* DNA from the plasmid recombinant *in vitro* and constructed a hybrid vector by joining plasmid pBR322-*amiE* (pCL28) to an IncQ group plasmid, pQSR136 (Meyer *et al.*, 1979). IncQ plasmids have been developed as cloning vectors for

use in *Pseudomonas* and related organisms (Bagdasarian *et al.*, 1981; Bagdasarian & Timmis, 1982; Hennam *et al.*, 1982). These plasmids are generally about 10 kb in size, are non-conjugative, have a copy number of 10–15 and replicate in both *E. coli* and *P. aeruginosa* (Grinter & Barth, 1976). The hybrid plasmids were used for complementation studies in *P. aeruginosa*.

METHODS

Bacteria. The bacterial strains used are listed in Table 1 (*a* and *b*).

Plasmids. The plasmids used are listed in Table 2.

Media. The *E. coli* strains were grown in L broth (Lennox, 1966) supplemented where necessary with streptomycin (Sm) (50 µg ml⁻¹), ampicillin (Ap) (50 µg ml⁻¹), tetracycline (Tc) (20 µg ml⁻¹). The *P. aeruginosa* strains were grown in Oxoid no. 2 nutrient broth supplemented as necessary with streptomycin (250 µg ml⁻¹) and/or carbenicillin (250 µg ml⁻¹).

For amidase assays the growth medium for *P. aeruginosa* was the basal medium of Brammar & Clarke (1964) with ammonium sulphate (0.1%, w/v) as nitrogen source and sodium succinate (1%, w/v) or sodium lactate (1%, w/v) as carbon sources. Lactamide (0.2%, w/v) was added for the inducing medium and butyramide (0.2%, w/v) for the repressing medium (Clarke, 1972). The minimal medium was supplemented with streptomycin (250 µg ml⁻¹) and carbenicillin (250 µg ml⁻¹) as appropriate.

The medium for growth of *E. coli* was the basal medium of Clarke & Laverack (1983) containing glucose (0.5%, w/v) or sodium succinate (0.5%, w/v) as additional carbon source. This was supplemented with amino acids at 0.003% (w/v) for growth of strain JA221. Inducing and repressing amides were added as for *P. aeruginosa*, and Sm and Ap (both at 50 µg ml⁻¹) were added as appropriate.

Table 1. *Bacterial strains*

(a) *E. coli*

Strain	Genotype (relevant features)	Reference
HB101	<i>hsdR recA rpsL20</i>	Rodriguez <i>et al.</i> (1977)
JA221	<i>hsdR recA</i>	Clarke & Carbon (1978)
C600	<i>thr leu thi</i>	Harayama <i>et al.</i> (1980)

(b) *P. aeruginosa*

Strain	Genotype	Amidase phenotype	Reference
PAO1161	<i>leu38 r⁻m⁺</i>	—	Dunn & Holloway (1971)
PAC1	<i>amiR⁺ amiE⁺</i>	Inducible Ami ⁺	Kelly & Clarke (1962)
PAC308	<i>amiR⁺ amiE8</i>	AmiE ⁻	Brammar <i>et al.</i> (1967)
PAC111	<i>amiR11 amiE⁺</i>	Constitutive Ami ⁺	Brammar <i>et al.</i> (1967)
PAC324	<i>amiR11 amiE20</i>	AmiE ⁻	Betz <i>et al.</i> (1974)
PAC142	<i>amiR33 amiE⁺ crp7</i>	Constitutive Ami ⁺ CRP ^R	Smyth & Clarke (1975a)
PAC326	<i>amiR33 amiE34 crp7</i>	AmiE ⁻	Betz & Clarke (1972)
PAC438	<i>amiR11,125 amiE⁺</i>	Ami ⁻ 41 °C, Ami constitutive 28 °C	Farin & Clarke (1978)
PAC623	<i>amiR223 amiE16</i>	AmiR ⁻ AmiE B ⁺	Farin & Clarke (1978)
PAC452	<i>ami Δ161</i>	Ami deletion	Day (1975)

Table 2. *Plasmids.*

Plasmid	Size (kb)	Markers*	Reference
pBR322	4.4	Ap Tc	Bolivar <i>et al.</i> (1977)
pJB950	9.1	Ap Ami	Clarke <i>et al.</i> (1981)
pCL28	6.9	Ap Ami	This paper
pQSR136	11.3	Sm	Meyer <i>et al.</i> (1979)
pCL31	18.2	Sm Ap Ami	This paper
pCL34	18.2	Sm Ap Ami	This paper
pTH10	60.4	Ap Km Tc	Harayama <i>et al.</i> (1980)

* The phenotypic abbreviations are for resistance to ampicillin, tetracycline, streptomycin, kanamycin and amidase-positive character.

Plasmid DNA preparations. (i) Large-scale DNA preparations were made using the sheared lysate method of Bazaral & Helinski (1968). (ii) In some of the experiments transformed colonies were examined for the presence of plasmid DNA by a modified version of the method of Goodman & MacDonald (1979). Overnight cultures (1 ml) in broth were pelleted, drained and frozen at -70°C . Frozen pellets were resuspended in 100 μl spheroplast forming mixture and lysed by addition of 50 μl 2% Sarkosyl (Bazaral & Helinski, 1968). After a clearing spin the supernatant was subjected to RNAase treatment, extracted with phenol/chloroform and the DNA ethanol precipitated and dried. DNA was dissolved in water and subjected to restriction enzyme analysis. (iii) In later experiments plasmid DNA was isolated from transformed cultures of *E. coli* and *P. aeruginosa* by the method of Birnboim & Doly (1979).

Enzymes and chemicals. Restriction endonucleases *Hind*II, *Hpa*II, *Pst*I, *Sal*I and λ DNA were purchased from Miles Laboratories, Stoke Poges, Slough, UK. *Eco*RI, *Hind*III, *Sma*I and *Taq*I were purchased from BCL, Lewes, UK. *Ava*I, *Kpn*I, *Sst*II, *Xho*I and *Xor*II were purchased from BRL. T4 DNA ligase was from CP Laboratories, Bishop's Stortford, UK. Most chemicals were AR grade. Caesium chloride was from BDH and HGT Agarose was from Miles Laboratories.

Endonuclease digestion and gel electrophoresis. Endonuclease digestions were carried out as prescribed by the manufacturer. Agarose gel electrophoresis was carried out as described by Clarke *et al.* (1981) using a Tris/borate buffer system (Peacock & Dingman, 1969) and gels were photographed using Polaroid 4 $\times$ 5 Landfilm type 55.

Ligase reactions. Restricted DNA to be ligated was mixed together and ethanol precipitated in 2% (w/v) sodium acetate at -70°C . Samples recovered by centrifugation were dried under vacuum and resuspended in 10 mM-Tris/HCl, pH 7.5, 100 mM-NaCl. Concentrated ligase cocktail was added and the DNA ligated overnight at 14°C as described previously (Drew *et al.*, 1980).

Transformations. Samples of plasmid DNA were used to transform *E. coli* strain JA221 made competent by the method of Mandel & Higa (1970) as described previously (Drew *et al.*, 1980). After growth in L broth for 90 min to allow plasmid genes to be expressed portions were overlaid in soft agar onto L plates containing antibiotics.

P. aeruginosa strains were made competent by a modification of the above procedure. Cultures were grown, harvested and resuspended in 0.5 vol. 0.1 M-MgCl₂. The cells were harvested, resuspended in 0.2 vol. 0.1 M-MgCl₂ and iced for 20 min. Finally, the cells were harvested and resuspended in 0.04 vol. 0.1 M-CaCl₂ and stored on ice for 60 min prior to DNA additions. Treatment was then as for *E. coli*.

Conjugation and mobilization using plasmid RP4. Approximately 1×10^8 donor and recipient cells from overnight cultures in broth were patch mated on L plates for 4 h at 30°C . Dilution buffer (0.2 ml per plate) was added and the cells were lifted off with a sterile glass spreader and pipette. The cells were pelleted, washed and serial dilutions plated onto appropriate selective plates at the desired temperature.

Amidase assays. Amidase was assayed in intact cells by the transferase assay (Brammar & Clarke, 1964) using acetamide as substrate. Bacterial growth was measured by absorption at 670 nm. Amidase specific activities are expressed as μmol acetylhydroxamate produced min^{-1} (mg bacteria) $^{-1}$.

RESULTS

Construction of plasmids pCL31 and pCL34

Plasmid pJB950 (Clarke *et al.*, 1981) is a derivative of pBR322 containing a 5.1 kb insert of *P. aeruginosa* PAC433 DNA between the *Hind*III and *Sal*I targets (Fig. 1*a*). Partial DNA sequence analysis has shown that the amidase N-terminal coding sequence begins 244 bp from the *Hind*III target (W. J. Brammar, personal communication). From the size of the amidase protein, approximately 340 amino acids, the coding region cannot extend beyond 1.3 kb from the *Hind*III site. To reduce the size of pJB950 and remove non-essential PAC433 DNA, pJB950 was digested with *Xho*I, ligated and transformed into *E. coli* HB101 with selection for ampicillin resistance. Thirty resistant colonies were picked and checked for amidase activity on solid medium (Drew *et al.*, 1980). Plasmid DNA was isolated from ampicillin-resistant, amidase-positive clones. Two clones carrying plasmids smaller than pJB950 were investigated further. Plasmid DNA from these was subjected to restriction enzyme analysis with *Ava*I, *Hind*III, *Kpn*I, *Pst*I, *Sal*I, *Sma*I, *Sst*II, *Xho*I and *Xor*II. Analysis showed that in both of these isolates the DNA between the *Xho*I targets at 2.38 and 4.23 kb on pJB950 had been deleted and in addition the *Xho*I target was not reconstituted (data not shown). One of these two isolates, pCL28, composed of approximately 2.7 kb from pBR322 and 3.1 kb of PAC433 DNA, was utilized further (Fig. 1*b*).

To construct a broad host range plasmid carrying the amidase structural gene, pCL28 DNA isolated from *E. coli* HB101 and pQSR136 DNA from *P. aeruginosa* PAO1162 were linearized

Fig. 1. Restriction maps of pBR322/*ami* plasmids. (a) Map of pJB950 (Clarke *et al.*, 1981) linearized from single *Hind*III target. Positions of genes and *Hind*III (H), *Xho*I (X), *Sal*I (S) and *Eco*RI (E) targets are to scale (kb). (b) Map of pCL28 showing *in vitro* constructed deletion of *Xho*I fragments 2.38 to 4.23 kb. The *Xho*I target (X) has not been reconstituted.

Fig. 2. Construction of plasmids pCL31 and pCL34. (a) Map of pCL28 with approximate gene locations and *Hind*III (H), *Sal*I (S) and *Eco*RI (E) targets. (b) Map of pQSR136 showing location of streptomycin resistant determinant and *Eco*RI (E) and *Hind*III (H) targets. (c, d) Maps of pCL31 and pCL34 showing gene locations and restriction enzyme targets.

with *Hind*III, ligated and used to transform *E. coli* JA221 to ampicillin/streptomycin resistance. Resistant clones were isolated at a frequency of 86 per μ g donor DNA. Plasmid DNA isolated from 12 of these transformants was analysed by digestion with *Hind*III, *Sal*I and *Eco*RI. Two isolates were identified as carrying hybrid plasmids in opposite orientations (Fig. 2): pCL31 and pCL34 are distinguished by the asymmetric location of *Eco*RI targets.

Transformation studies with plasmids

Transformation of *E. coli* JA221 with plasmids of increasing sizes showed a reduction in transformation frequency (Table 3) as size increased. pBR322 (4.4 kb) yielded 2.2×10^6 transformants per μ g DNA; pCL28 (6.9 kb) yielded 8.4×10^5 transformants per μ g DNA; and pQSR136 (11.3 kb) yielded 8.0×10^4 transformants per μ g DNA. *E. coli* JA221 appeared to be a better transformation recipient than strain HB101 by an order of magnitude. *P. aeruginosa* was

Table 3. Transformation frequencies of plasmids

Plasmid	Size (kb)	Host	Recipient	Selection*	No. transformants per μg DNA
pBR322	4.4	HB101	JA221	Ap	2.2×10^6
pBR322	4.4	HB101	HB101	Ap	3.6×10^5
pCL28	6.9	HB101	JA221	Ap	8.4×10^5
pQSR136	11.3	PAO1162	JA221	Sm	8.0×10^4
pQSR136	11.3	PAO1162	PAC308	Sm	8.2×10^2
pQSR136	11.3	PAO1162	PAC623	Sm	3.5×10^3
pQSR136	11.3	PAO1162	PAO1162	Sm	9.9×10^2
pCL31	18.2	JA221	PAC308	Cb Sm	0
pCL34	18.2	JA221	PAC308	Cb Sm	0
pCL31	18.2	JA221	PAO1162	Cb Sm	0
pCL34	18.2	JA221	PAO1162	Cb Sm	1.1×10^2

* Antibiotic selection was for resistance to ampicillin, streptomycin and carbenicillin.

Table 4. Amidase activity in *E. coli*

Cells were incubated overnight at 37 °C and amidase activity was measured in intact cells by the transferase assay (Brammar & Clarke, 1964). Amidase activity is expressed as μmol acethydroxamate formed min^{-1} (mg bacteria) $^{-1}$. Values expressed are the mean of three experiments.

Strain	Plasmid	Growth conditions:				
		Glucose	Glucose/ lactamide	Succinate	Succinate/ lactamide	Succinate/ butyramide
JA221	—	0.05	0	0	0	0
JA221	pCL28	0.22	0.24	0.11	0.14	0.14
JA221	pCL31	0.79	0.82	0.89	1.03	0.92
JA221	pCL34	0.42	0.49	0.19	0.17	0.24

transformed much less readily than *E. coli* by the broad host range Q group plasmid. Frequencies were reduced by 100-fold for pQSR136 transformed into PAC or PAO strains (Table 3). Attempts to transform pCL31 and pCL34 directly into PAC strains with selection for carbenicillin/streptomycin resistance were unsuccessful. Strain PAO1161 has been described as a good recipient for transformation and contains the conjugative plasmid FP2. pCL31 and pCL34 were transformed into strain PAO1161 with drug resistance as the selection. Only pCL34 transformants were obtained at a frequency of 1.1×10^2 per μg DNA. Agarose gel electrophoresis of mini plasmid DNA preparations confirmed the presence of pCL34 (data not presented).

Amidase expression in *E. coli* strains

Amidase expression in *E. coli* JA221 carrying various *ami* plasmids was measured under inducing, non-inducing and repressing conditions (Table 4). Wild-type *E. coli* normally shows no growth on acetamide medium and gives little or no colour reaction in the transferase assay. Strain JA221 carrying pCL28 showed low constitutive amidase expression under all growth conditions. This suggested that the *amiR* gene was absent or non-functional. There was no evidence to indicate if this amidase expression originated from the amidase promoter or from the remnants of the pBR322 tetracycline resistance promoter at the *Hind*III join. *E. coli* JA221 containing the hybrid plasmids pCL31 and pCL34 showed higher levels of amidase activity (Table 4). Amidase expression was constitutive and not subject to induction or repression. Expression from pCL34 was approximately twofold higher than from pCL28, and that from pCL31 between five- and tenfold higher. These increases were not investigated further but it was envisaged that the increased activity was due to read-through transcription from promoters located in pQSR136.

Table 5. *Amidase expression in P. aeruginosa*

Cells were incubated overnight at 37 °C unless otherwise indicated. Amidase activity was measured in intact cells using the transferase assay (Brammar & Clarke, 1964). Amidase activity is expressed as μmol acethydroxamate formed min^{-1} (mg bacteria) $^{-1}$. The results are means of at least 3 experiments and the variation was $\pm 15\%$.

No.	Strain	Isolate	Phenotype*	Plasmid pCL34†	Growth conditions:				
					Succinate	Succinate/ lactamide	Lactate	Lactate/ lactamide	Lactate/ butyramide
1	PAC1		Ind Ami ⁺	—	0.09	8.11	0.14	3.94	0.08
2	PAC308		Ind Ami ⁻	—	0.05	0.06	0.09	0.07	0.06
3	PAC308		Ind Ami ⁻	+	0.81	24.10	0.98	8.74	0.37
4	PAC111		Con Ami ⁺	—	9.75	9.90	10.45	9.51	0.08
5	PAC324		Con Ami ⁻	—	0.21	0.15	0.11	0.13	0.19
6	PAC324		Con Ami ⁻	+	28.87	28.39	25.39	25.28	6.83
7	PAC142		Con Ami ⁺ Crp ^R	—	28.48	24.75	31.10	31.70	22.21
8	PAC326		Con Ami ⁻ Crp ^R	—	0.18	0.19	0.40	0.65	0.61
9	PAC326	5	Con Ami ⁻ Crp ^R	+	142.29	111.23	116.07	95.04	100.96
10	PAC326	6	Con Ami ⁻ Crp ^R	+	49.53	52.88	58.03	54.24	52.59
11	PAC326	10	Con Ami ⁻ Crp ^R	+	32.00	32.05	20.76	20.72	12.59
12	PAC438		Reg ts 42 °C‡	—	0.06	0.10	0.17	0.23	0.21
13	PAC438		Reg ts 42 °C	+	1.03	1.61	1.52	1.92	0.76
14	PAC438		Reg ts 26 °C	—	5.37	2.76	3.29	2.26	0.26
15	PAC438		Reg ts 26 °C	+	8.91	4.40	5.74	4.92	1.75
16	PAC452		AmiREA	—	0	0.07	0.07	0.12	0
17	PAC452		AmiREA	+	2.39	2.27	3.41	3.58	1.60
18	PAC623		Reg ⁻ Ami ⁺ B§	—	0	0.08	0.01	0	0.02
19	PAC623		Reg ⁻ Ami ⁺ B	+	3.87	3.09	2.54	2.24	1.69

* Ind, inducible; Con, constitutive; Reg ts, temperature-sensitive regulator.

† Indicates the presence (+) or absence (—) of plasmid pCL34.

‡ Growth temperature for regulator temperature-sensitive strain; assays were carried out at 37 °C.

§ PAC623 is *amiR* and carries the B6 mutation in *amiE* which shows greater activity towards butyramide as enzyme substrate (Farin & Clarke, 1978).

Plasmid mobilization experiments

To study complementation of amidase, attempts were made to mobilize plasmids pCL31 and pCL34 into *P. aeruginosa* PAC strains carrying various structural and regulator gene mutations. Attempts to use strain PAO1162(FP2, pCL34) as donor into strain PAC308 *amiR*⁺ *amiE* were unsuccessful. It has been reported that mobilization of IncQ group plasmids by FP2 is of the order of 1×10^{-7} (Willets & Crowther, 1981) and restriction systems in the recipient (unpublished observations) would be expected to reduce this further.

This was overcome by using the broad host range plasmid RP4 to mobilize pCL31, pCL34 from *E. coli* to *P. aeruginosa*. A derivative of RP4, pTH10, that was temperature-sensitive for replication, was used to facilitate loss of the mobilizing plasmid from the transconjugants. Plasmid pTH10 was transferred into strains JA221(pCL31) and JA221(pCL34) from *E. coli* C600 by plate mating. Transconjugants were obtained and shown to contain both plasmids by antibiotic resistance determinations and restriction enzyme analysis of plasmid DNA isolations (data not presented).

Strains JA221(pTH10, pCL31) and JA221(pTH10, pCL34) were used as donors to mobilize the non-conjugative plasmids into *P. aeruginosa* PAC308. Antibiotic selection gave transconjugants from the JA221(pCL34) cross only. *Hind*III and *Sal*I restriction enzyme analysis of plasmid DNAs isolated from the transconjugants showed that only pCL34 was present after subculturing at 37 °C. The successful donor in this experiment was used to mobilize pCL34 into various other PAC recipients. By this means pCL34 was transferred into strains PAC324, PAC326, PAC438, PAC623 and PAC452. Restriction enzyme analysis of plasmid DNA isolated from transconjugants from all of these crosses showed the presence of only one plasmid corresponding to pCL34.

Complementation studies

Gene expression studies from plasmids pCL28, pCL31 and pCL34 in *E. coli* had indicated that plasmid pCL34 was functionally *amiR*: deletion, *amiP*: constitutive, *amiE*: positive. Amidase activity was measured in *P. aeruginosa* transconjugants under inducing, non-inducing and repressing conditions. Cells were grown in minimal medium with succinate or lactate as carbon sources. Lactamide and butyramide were added as inducer or amide analogue repressor, respectively. These two amides are hydrolysed poorly by amidase, and enzyme activity was not required for bacterial growth.

Under these conditions wild-type and mutant *P. aeruginosa* strains showed their expected phenotypes (Table 5). Strain PAC1 was induced by lactamide (line 1), PAC111 showed constitutive enzyme synthesis which was repressed by butyramide (line 4) and PAC142 showed high constitutive synthesis due to the unlinked mutation conferring resistance to catabolite repression (line 7). Strain PAC438, which contains a regulator (*amiR*) temperature-sensitive mutation, showed low constitutive enzyme synthesis at 26 °C, which was sensitive to butyramide repression, and no enzyme activity at 42 °C (lines 12 and 14).

Amidase activity was lacking in the *amiE* derivatives of these strains, PAC308, PAC324, PAC326 and also in PAC452 and PAC623. Strain PAC452 had previously been characterized as containing a deletion of at least the amidase structural gene and PAC623 is an *amiR* strain derived from an inducible strain which produces the mutant B amidase.

Amidase expression from plasmid pCL34 in strain PAC308 was controlled by the chromosomally located *amiR* gene product (line 3). Under non-inducing conditions (succinate or lactate) low amidase expression was detectable, which was somewhat repressed by growth in the presence of butyramide. Under inducing conditions (succinate/lactamide or lactate/lactamide) high levels of amidase activity were observed: three times that of the fully induced wild-type under similar conditions.

Constitutive amidase expression from pCL34 was found in hosts carrying mutant constitutive *amiR* genes: PAC324 (line 6) and PAC326 (lines 9–11). Activity was measured in six independently isolated transconjugants of PAC324 all of which showed activities some threefold higher than the parental strain PAC111 (line 4). This activity was partially butyramide repressible, showing control by the chromosomal *amiR* gene product. Greater variation was

Fig. 3. Derivation of amidase-negative mutants from constitutive parents. Amidase-specific activities are given as μmol acetylhydroxamate produced min^{-1} (mg bacteria) $^{-1}$. Amidase activity was measured in whole cells after overnight growth in succinate basal medium (see Methods).

found in isolates of strain PAC326 containing pCL34. Three such isolates gave specific activities between 20 and 100 (lines 9–11) compared with the parental strain PAC142 whose specific activity ranged from 22 to 32 under these growth conditions. This finding was unexpected since the isolates were thought to be identical. However, these values were found to be reproducible on retesting.

Expression from plasmid pCL34 in the temperature-sensitive regulator mutant PAC438 at 42 °C (line 13) showed low regulator-independent expression that was repressed slightly by growth on butyramide. Expression at 26 °C was presumed to be from both plasmid and chromosomal *amiE* genes. The amidase activity of strain PAC438 at 26 °C was doubled in the presence of plasmid pCL34 (lines 14 and 15). However, the specific activities were reduced by growth in medium containing butyramide, since the thermolabile regulator in this strain is sensitive to butyramide repression.

There was some amidase synthesis from plasmid pCL34 in *P. aeruginosa* strains thought to lack a functional *amiR* product. Expression from pCL34 in PAC452 and PAC623 (lines 17 and 19) was constitutive under all growth conditions at levels some 20–40% of the fully induced wild-type PAC1. Since PAC623 had been genetically defined as an *amiR* mutant and PAC452 as a deletion, amidase expression was presumed to result from interactions of host RNA polymerase with either the amidase promoter of pCL34 or the up-stream pQSR136 sequences.

The results of the complementation analysis shown in Table 5 allowed undefined amidase-negative mutations to be located. Complementation with pCL34 which gave high constitutive activity located the *ami* mutations of strains PAC324 and PAC326 into the structural gene *amiE*. Similarly, the low activities with pCL34 in strains PAC323 and PAC327 indicated that regulator gene mutations had caused the amidase-negative phenotype (Fig. 3).

Analysis of pCL34 transconjugants

It was considered that the different levels of amidase activity seen in PAC326(pCL34) isolates might have resulted from recombination. To examine this possibility, plasmid-free segregants were obtained from isolates 5 and 10 (see Table 5). A small number of cells were used to inoculate antibiotic-free minimal medium and the inoculum was grown overnight. Serial dilutions of the overnight culture were plated on solid medium with or without antibiotics to measure the frequency of plasmid loss. Plasmid-free segregants were isolated at high frequency and tested for amidase activity. Less than 1 in 300 colonies screened retained pCL34 after non-selective growth from both isolates. Five independent colonies from isolates 5 and 10 were tested and all had specific activities < 1.0 under all growth conditions. Thus the different activities exhibited by

Fig. 4. Map showing location of *amiE* gene with respect to Tn5 promoters in plasmids pCL31 (a) and pCL34 (b). Neither the size of genes nor the size of inverted repeats or the distances between them are drawn to scale. Symbols: ~, pQSR136 DNA; —, pCL28 DNA; □, inverted repeats of Tn5; ■, location of genes on pCL28; ←, indicates origin and direction of transcription. (1) Transposition function transcripts and (2) transcription from neomycin phosphotransferase II promoter. Tn5 information from Rothstein *et al.* (1980). Location of *Hind*III (H) and *Eco*RI (E) restriction enzyme targets are shown.

isolates PAC326(pCL34/5) and PAC326(pCL34/10), of 100 and 26, respectively, could not be explained by recombination between the plasmid-carried up-promoter/structural gene and the chromosomal sequences but may have been due to differences in copy number of their plasmids.

DISCUSSION

Complementation analysis using cloned genes offers an alternative to R' or F' plasmid isolation in bacterial species where R' isolation is difficult. Cloned genes offer the advantages of being physically defined by restriction enzyme mapping and have the potential for *in vitro* manipulations to locate DNA sequences of importance. Thus specific deletions and insertions may be constructed to investigate the sequences controlling gene expression. The hybrid plasmid pCL34 which is able to replicate in both *P. aeruginosa* and *E. coli* carries a fragment of the *P. aeruginosa* chromosome containing the amidase structural gene and an up-promoter mutation, but lacks a functional regulator activity. Transduction analysis of the amidase genes had shown about 90% linkage between the structural and regulator genes (Brammar *et al.*, 1967), but the lack of nearby markers meant that the gene order was unknown.

Amidase expression from pCL28 in *E. coli* was at low constitutive levels under all growth conditions, showing the absence of regulated gene expression. It is proposed that this expression results from low-affinity interaction between RNA polymerase and either the amidase promoter or the remnants of the tetracycline resistance promoter at the *Hind*III join. *In vitro* transcription studies have indicated that *E. coli* RNA polymerase can initiate transcription from a restriction enzyme fragment containing just the *P. aeruginosa* DNA sequences (C. Turberville, personal communication).

Higher constitutive levels of enzyme activity were seen from the hybrid replicons pCL31 and pCL34 in *E. coli*. Expression from pCL31 was five times greater than that from pCL28 and expression from pCL34 was twice as great as that from pCL28. In plasmids pCL31 and pCL34 the *amiE* gene was located downstream from Tn5 sequences present in pQSR136. The different levels of constitutive amidase expression from the two hybrids was thought to result from read-through transcription from Tn5 promoters (Fig. 4). Tn5 has been investigated with regard to expression of transposition functions and neomycin phosphotransferase (NPT) II activity and the promoters for these functions have been identified (Rothstein *et al.*, 1980). However, it is not readily apparent from this information how read-through transcription could account for the different amidase activities in *E. coli*. In pCL31, which shows the higher activity, the amidase promoter lies back-to-back with the NPT II promoter which is itself part of the non-homologous inverted repeat of Tn5. In pCL34 transcription originating at the left end of the inverted repeat and running into the amidase gene could explain the enhanced expression compared with pCL28.

Expression studies from pCL34 in *P. aeruginosa* PAC strains confirmed the lack of amidase regulator function on the cloned fragment. The presence of a functional *amiR* gene in the *cis* configuration would be expected to show very high amidase expression similar to the parental strain PAC433 which has a specific activity of >50. Instead, in the absence of functional R protein (PAC623, pCL34) low constitutive enzyme levels are found. These low levels were an average of ninefold higher in *P. aeruginosa* *amiR* strains than in *E. coli*, presumably reflecting increased RNA polymerase/promoter interactions.

Under inducing conditions with PAC308(pCL34) and non-inducing conditions with PAC324(pCL34) and PAC326(pCL34) complementation was observed. For this to occur it is apparent that the regulator protein must function in *trans*. The results show dominance of regulator-inducible or regulator-constitutive over regulator-negative and confirm the positive control model for amidase expression proposed by Farin & Clarke (1978). The cloned *amiR* *amiP*^{con} *amiE*⁺ fragment is complemented by chromosomally located *amiR*⁺ *amiP*⁺ *amiE* and also by *amiR*^{con} *amiP*⁺ *amiE* with the predicted results. Complementation studies with pCL34 allowed previously uncharacterized amidase-negative mutations to be assigned to the regulator or structural genes, respectively.

However, these studies suggested that amidase regulation may be somewhat more complex than a simple positive control. Amidase synthesis in *amiR* hosts carrying pCL34 was at a low constitutive level, but in *amiR*⁺*E* hosts under non-inducing or repressing conditions expression from the plasmids was further reduced (Table 5). This may be taken to indicate that the regulator protein has a dual function. With the wild-type system, in the absence of inducer, the regulator may act as a repressor to reduce *amiE* transcription to a very low level. During induction the regulator protein combined with inducer stimulates *amiE* transcription to a high level.

Dominance relationships between various constitutive and inducible alleles now being investigated may elucidate this aspect of amidase regulation.

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