Genet. Res. Camb. (1986), **47**, *pp.* 93–102 *With 3 text-figures Printed in Great Britain*

Phosphatase regulation in *Aspergillus nidulans*: responses to nutritional starvation

MARK X. CADDICK*, ALAN G. BROWNLEE† AND HERBERT N. ARST, JR*‡

Department of Genetics, Ridley Building, The University, Newcastle upon Tyne NE1 7RU, England

(*Received 26 September 1985 and in revised form 12 November 1985*)

Summary

The regulation of the syntheses of a number of phosphatases in the fungus *Aspergillus nidulans* has been examined. Levels of the intracellular alkaline phosphatase PII are increased by starvation for carbon, nitrogen, phosphorus or sulphur. There is, however, no evidence that any of the wide domain regulatory genes which mediate sufficiency-triggered repression for each of these elements is involved. A possible interpretation is that all four forms of starvation result in accumulation of an inducing metabolite. The *palc*A gene has been identified as a wide domain, probably positive-acting regulatory gene mediating phosphate repression. The *palc*A product controls the syntheses of alkaline phosphatase PI, acid phosphatases PIII and PV, a phosphodiesterase lacking phosphomonoesterase activity and probably also a phosphate permease. Mutations resulting in derepression of phosphate-repressible activities at acid but not alkaline growth pH define a gene designated *pac*J. *pac*J mutations also confer arsenate resistance at low but not high pH. It is likely that phosphate derepression and arsenate resistance result from reduced uptake of $H_2PO_4^-$. Finally, phosphatase regulation might be less complex than previously thought. Mutations designated *r* and mapping at several loci apparently have no effect on phosphatase. They enhance phosphatase colony staining but this occurs even if the phosphatase substrates are omitted from the staining mixtures. *r* mutations appear to promote reactions converting the diazonium salts used for phosphatase staining to coloured precipitates.

1. Introduction

Early work suggested that phosphate assimilation in the ascomycete fungus *Aspergillus nidulans* might be a very complex process involving a large number of genes (Dorn, 1965*a*, *b*). In a previous report (Caddick & Arst, 1986) structural genes for three or possibly four of the phosphatases of *A. nidulans* were identified. Here some of the factors controlling expression of these and other, as yet unidentified, structural genes involved in phosphate metabolism are examined.

2. Materials and Methods

(i) *Genetic techniques, growth testing and strains*

Genetic techniques were modified after Pontecorvo *et al.* (1953), McCully & Forbes (1965) and Clutterbuck (1974). Growth testing of *A. nidulans* has been described by Arst & Cove (1969) and Arst, Tollervey & Sealy-Lewis (1982). The solid complete and minimal media of Cove (1966) were used. This minimal medium is phosphate-sufficient and its pH is 6·5. Medium lacking phosphate ($-P_i$ medium) differs from minimal medium in that chloride salts replace phosphate salts at equimolar cation concentrations. Solid minimal media of other than pH 6.5 were the pH 5 medium and the phosphate-buffered pH 8 medium of Cove (1976). All of these media contained (final concentrations) 1% (w/v) D-glucose as carbon source and, unless otherwise specified, 10 mM ammonium (as the (+)−tartrate) as nitrogen source. Unless otherwise stated, a growth temperature of 37 °C was used.

Markers carried by *A. nidulans* strains have been described previously (Clutterbuck, 1984 and references therein; Caddick & Arst, 1986) with the exception listed below. *palc*A-40 was selected after UV mutagenesis of a strain of genotype *palc*A-1 (*p*-aminobenzoate-requiring) as failing to stain for acid phosphatase activity on $-P_i$ medium to which (final concentration) 100 μM phosphate was added. It is recessive to *palc*A$^+$ and fails to complement *palc*A-1 in diploids. In

* Present address: Department of Bacteriology, Royal Postgraduate Medical School, Hammersmith Hospital, Ducane Road, London W12 0HS, England.
† Present address: CSIRO Division of Animal Production, P.O. Box 239, Blacktown, NSW 2148, Australia.
‡ Address correspondence to this author.

a cross of a *palc*A-40 strain to a *palc*A-1 strain no wild-type recombinants were obtained out of more than 1000 progeny. *palc*A-40 has a similar but more extreme phenotype than *palc*A-1 (Dorn, 1965*a*, *b*). It can be concluded that the two mutations are allelic.

*pac*J-121, -122, -123 and -124 were selected after 4-nitroquinoline-1-oxide (NQO) mutagenesis (Bal, Kajtaniak & Pieniazek, 1977) of a strain of genotype *paba*A-1 as conferring resistance to 1 mM arsenate (added as Na_2HAsO_4) in the presence of 1% (w/v) β-glycerophosphate (disodium salt) as phosphate source in appropriately supplemented $-P_i$ medium buffered with (final concentration) 50 mM citrate (sodium salt) at pH 4·6. They are recessive to *pac*J$^+$ in heterozygous diploids. *pac*J-122, -123 and -124 are tightly linked (< 1 cM) to *pac*J-121 and fail to complement it in diploids.

*r*D-30 was selected after UV mutagenesis of a strain of genotype *bi*A-1 (biotin-requiring) *palc*A-10 (lacking phosphate-repressible phosphatases) *pu*A-2 (putrescine-requiring) *cha*A-1 (chartreuse conidial colour) as leading to staining for phosphodiesterase activity (Caddick & Arst, 1986) after growth for 2 days at 37 °C on appropriately supplemented $-P_i$ medium to which (final concentration) 100 μM phosphate was added. *r*E-40 was selected after NQO mutagenesis of a strain of genotype *paba*A-1 as resulting in phosphodiesterase staining after growth on appropriately supplemented minimal (high phosphate) medium containing 0·4% (w/v) sodium deoxycholate for 36 h at 37 °C. Both *r*D-30 and *r*E-40 are recessive to their wild-type alleles in heterozygous diploids.

(ii) *Colony staining*

Staining colonies for acid and alkaline phosphatases followed the method of Dorn (1965*a*) as modified by Caddick & Arst (1986). Colony staining for phosphate-repressible phosphodiesterase has been described previously (Caddick & Arst, 1986).

(iii) *Starch gel electrophoresis*

Starch gel electrophoresis was carried out as described previously (Dorn, 1965*a*; Caddick & Arst, 1986). Culture media were concentrated approximately fourfold for electrophoresis by dialysis using Aquacide I-A (Calbiochem) at 4 °C. Gels were stained by flooding with the appropriate colony staining solution.

(iv) *Growth and harvesting of mycelia*

Mycelia were grown, harvested and stored as described previously (Cove, 1966; Arst, Brownlee & Cousen, 1982). The liquid minimal medium of Cove (1966) containing (final concentrations) 1% (w/v) D-glucose as carbon source and 20 mM ammonium (as the (+)-tartrate) as nitrogen source was used. Liquid medium lacking a sulphur source (−S medium) was prepared by substituting chloride salts for sulphate salts at equimolar cation concentrations. $-P_i$ liquid medium was prepared by substituting chloride salts for phosphate salts at equimolar cation concentrations. Strongly buffered media were made by adding (final concentrations) 50 mM citrate (sodium salt) at pH 5 and 6·5 and 50 mM tris HCl at pH 8. All strains used for enzyme assays or starch gel electrophoretic analysis carry *paba*A-1. The *xpr*D-1 strain also carries *bi*A-1. All liquid media were supplemented with 10 μg/l biotin and 4 mg/l *p*-aminobenzoate. Culture media for enzyme determinations were collected by filtration through Miracloth.

(v) *Extraction procedure and enzyme assays*

Preparation of cell-free extracts and assays for acid and alkaline phosphatases were described previously (Caddick & Arst, 1986). For phosphodiesterase assays 20 μl of cell-free extract or 50 μl of culture medium was made up to 1 ml in 100 mM acetate (sodium salt) buffer at pH 4·6 containing (final concentrations) 10 mM-$MgCl_2$ and (as indicated) 1 mM-*p*-nitrophenyl phenylphosphonic acid (PNPP-P) or 1 mM-*bis*-(*p*-nitrophenyl)-phosphoric acid (bis-PNP-P). The reactions were terminated by the addition of 2 ml of 100 mM-NaOH and *p*-nitrophenol was estimated from the absorbance at 400 nm. Soluble protein in extracts was determined by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as standard. Specific activities are expressed in nmoles *p*-nitrophenol liberated per mg soluble protein in extract (cell-free extracts) or per mg dry weight (culture media) both per minute at 30 °C. For comparison of intra- and extracellular enzyme levels, it should be noted that soluble protein in extracts accounts for approximately 12% of mycelial dry weight (Brownlee & Arst, 1983).

3. Results and Discussion

(i) *Levels of the alkaline phosphomonoesterase PII can be increased by starvation for carbon, nitrogen, phosphorus or sulphur*

The electrophoresis pattern in Fig. 1 shows that the level of the *pal*G-encoded intracellular alkaline phosphatase PII (Caddick & Arst, 1986) increases considerably in response to carbon, nitrogen, phosphorus or sulphur starvation. (See also figure 1 of Caddick & Arst, 1986). This can be seen quantitatively in Table 1*. As expected, the structural gene loss-of-function mutation *pal*G-21 prevents any increase in alkaline phosphatase levels in response to carbon, nitrogen or sulphur starvation. Phosphorus starvation, however, results in phosphate derepression of the

* For the purposes of this paper PII and PII′, both encoded by *pal*G (Caddick & Arst, 1986), are assumed to be regulated in identical fashion when bulk activities are measured.

https://doi.org/10.1017/S0016672300022916 Published online by Cambridge University Press

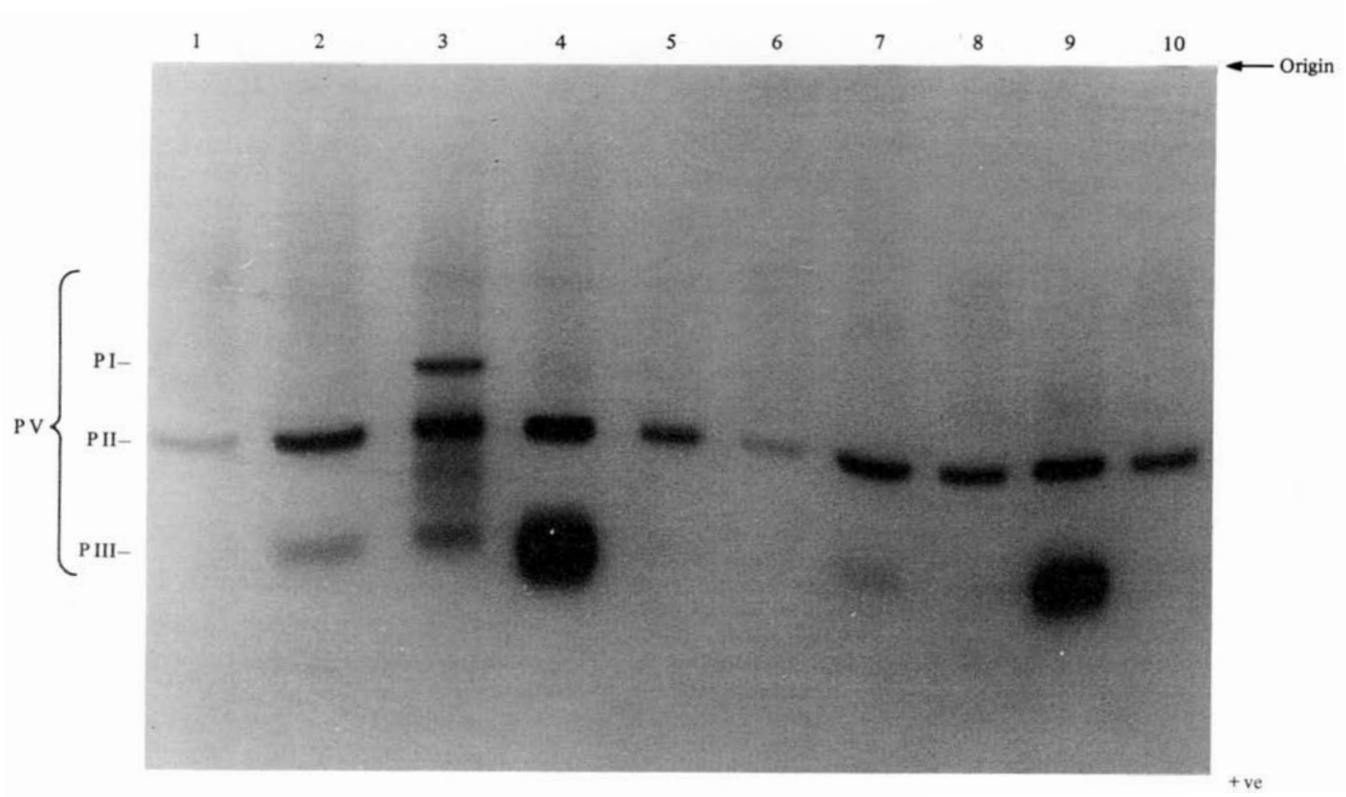

Fig. 1. Effects of a *palc*A⁻ mutation and nutrient starvation on starch gel electrophoresis patterns of acid and alkaline phosphatases. Mycelia were grown in appropriately supplemented shaken liquid minimal medium for 12 h at 37 °C (lanes 1 and 6) or additionally followed by 4 h in the same conditions but in nitrogen-free medium (lanes 2 and 7) or by 6 h in the same conditions but in phosphate-free medium (lanes 3 and 8) or by 6 h in the same conditions but in medium lacking a carbon source (lanes 4 and 9) or by 6 h in the same conditions but in medium lacking a sulphur source (lanes 5 and 10). Cell-free extracts were prepared from a wild type (*palc*A⁺) (lanes 1–5) and a *palc*A-40 (lanes 6–10) strain. The gel was stained at room temperature for alkaline phosphatase activity for 15 min and then (without washing) for acid phosphatase activity for 30 min.

Table 1. *Effects of nutrient starvation and various mutations on alkaline phosphatase activity*

Relevant genotype	Alkaline phosphatase				
	MM	−P	−N	−C	−S
Wild type	18·5±2·7	133±4	104±11	99·2±2·0	87·4±6·6
*pal*G-21	5·2±0·4	63·9±2·3	5·0±1·1	5·3±0·7	7·4±0·5
*pal*D-8	19·3±0·5	90·0±3·8	127±18	n.d.	n.d.
*pal*G-21 *pal*D-8	3·1±0·2	7·3±3·5	2·5±0·4	n.d.	n.d.
*palc*A-40	15·1±2·1	52·5±6·4	96·7±6·2	98·5±7·5	62·5±1·4
*palc*A-40 *pal*G-21	2·8±0·8	4·6±0·4	n.d.	n.d.	n.d.
*are*A^{r}-18	26·0±3·4	139±0·7	89·5±7·8	108±10	87·0±4·0
*are*A^{r}-18 *pal*G-21	2·4±0·3	n.d.	< 0·1	n.d.	n.d.
*xpr*D-1	19·4±1·3	115±8	102±11	n.d.	n.d.
*cre*A^{d}-1	23·3±2·4	n.d.	n.d.	77·8±4·9	n.d.

Cell-free extracts were prepared from mycelia grown for 12 h at 37 °C in appropriately supplemented shaken liquid minimal medium (MM) or additionally followed by 6 h in the same conditions in medium lacking phosphate (−P), a carbon source (−C) or a sulphur source (−S) or by 4 h in the same conditions in medium lacking a nitrogen source (−N). Specific activities ±1 S.D. are given. n.d., Not determined.

*pal*D-encoded (Caddick & Arst, 1986) alkaline phosphatase PI (Fig. 1). Elimination of the response to phosphorus starvation therefore requires loss-of-function mutations in both *pal*D and *pal*G (Table 1).

Data in Table 1 also show that at least in the cases of carbon, nitrogen and phosphorus, the response of PII to starvation is unlikely to be mediated by the wide domain regulatory genes (Arst, 1984; Arst & Scazzocchio, 1985) which control the syntheses of enzymes and permeases providing usable forms of these elements. Nitrogen metabolite repression is mediated by the positive-acting regulatory gene *are*A (Arst & Cove, 1973; Hynes, 1975; Al Taho, Sealy-Lewis & Scazzocchio, 1984; Wiame, Grenson & Arst, 1985).

https://doi.org/10.1017/S0016672300022916 Published online by Cambridge University Press

*are*A^r-18 is a complete loss of function mutation in which a translocation breakpoint occurs in the *are*A gene (Rand & Arst, 1977; Arst, 1981).

Although *are*A^r-18 leads to extremely low levels of nitrogen metabolite-repressible activities [e.g. Tollervey & Arst, 1981; Arst *et al.* 1982), it does not significantly affect the response of PII to nitrogen starvation. The absence of such a response in an *are*A^r-18 *pal*G-21 double mutant (Table 1) confirms that it is due to an increase in PII levels. *xpr*D-1 is an inversion-associated *are*A alle which leads to nitrogen metabolite derepression (Cohen, 1972; Arst, 1982). Howver, *xpr*D-1 does not affect alkaline phosphatase levels under nitrogen sufficient or nitrogen starvation growth conditions (Table 1). Carbon catabolite repression is mediated by the probably negative-acting regulatory gene *cre*A (Bailey & Arst, 1975; Arst & Bailey, 1977). The absence of any significant effect of *cre*A^d-1, which leads to carbon catabolite derepression (Bailey & Arst, 1975), shows that the response for PII to carbon starvation is unlikely to be mediated by *cre*A.

Phosphate repression is mediated by the probably positive-acting wide domain regulatory gene *palc*A- (see Section (iii) of Results and Discussion). In Fig. 1 it is evident that the loss-of-function mutation *palc*A-40 does not affect the response of PII to phosphorus starvation. The same conclusion can be reached from measurements of total alkaline phosphatase activity in Table 1. The increase in total alkaline phosphatase activity in the wild type upon phosphorus starvation is due to increases in both PI and PII (Fig. 1; Caddick & Arst, 1986). *palc*A-40 reduces but does not abolish the response of total alkaline phosphatase activity to phosphate starvation (Table 1). The remaining response is, however abolished in a *palc*A-40 *pal*G-21 double mutant. This shows, in agreement with Fig. 1, that an increase in the level of PII is responsible for the phosphate starvation response in the *palc*A-40 strain. The effect of phosphorus starvation on PII levels is therefore not under *palc*A control.

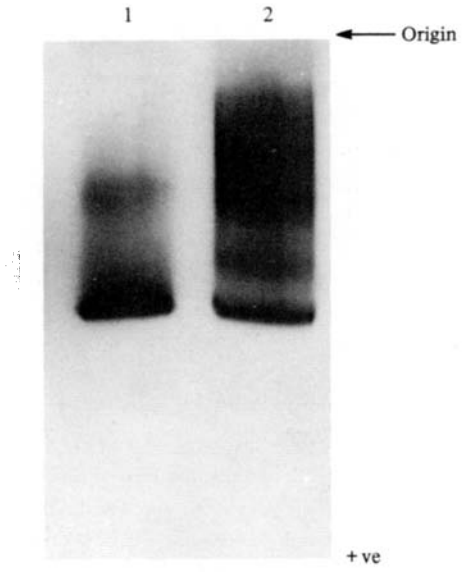

Fig. 2. Starch gel electrophoresis patterns of intracellular and secreted phosphodiesterases. Mycelia of a wild-type strain were grown for 15 h at 37 °C in appropriately supplemented shaken liquid phosphate-free medium to which (final concentration) 200 μM phosphate (Na^+ salts, adjusted to pH 6·5) was added. The gel was made in 40 mM glycine/40 mM tris HCl buffer at pH 8 and run in the same buffer made fivefold more concentrated. Phosphodiesterase activity was stained for 25 min at room temperature. Lane 1, culture medium; lane 2, cell extract.

The mechanism(s) of control of *pal*G expression remains obscure. The similarity of the responses of PII to carbon, nitrogen, phosphorus and sulphur starvation and the lack of involvement of *cre*A, *are*A and *palc*A might suggest that induction rather than repression is operative. One possibility is that an inducting metabolite(s) accumulates in any of these starvation conditions. It is also possible that starvation results in derepression through depletion of a common metabolite.

(ii) *Phosphodiesterase activity can be used as a particularly sensitive measure of phosphate repression*

A nitrogen metabolite-repressible phosphodiesterase-phosphomonoesterase enzyme in *A. nidulans* has been described by Polya, Brownlee & Hynes (1975). In addition the organism has a phosphate-repressible phosphodiesterase (Brownlee, Caddick & Arst, 1983). This latter enzyme exists in multiple electrophoretically and physically separable forms, both intra- and extracellularly (Fig. 2; A. G. Brownlee, unpublished data). Whether these forms are specified by a single or multiple structural genes is not known but it is possibly pertinent that our efforts to select structural gene mutations have been unsuccessful. Nevertheless, even if bulk phosphate-repressible phosphodiesterase activity results from multiple enzymes specified by multiple structural genes, the enzymatic properties and regulation of synthesis of these enzymes are sufficiently similar that they can be considered a single enzyme for present purposes.

Two features of the phosphate-repressible phosphodiesterase(s) are particularly convenient. One of these is its unusual substrate specificity. It is unable to catalyse the hydrolysis of any phosphomonoester tested, including the phosphomonoesterase assay substrate *p*-nitrophenylphosphate and staining substrate α-naphthyl acid phosphate. Thus it does not interfere with measurement of phosphomonoesterase activity. Moreover, it differs from the nitrogen metabolite-repressible phosphodiesterase-phosphomonoesterase in that it has relatively little activity with *bis*-(*p*-nitrophenyl)-phosphate (bis-PNP-P) as substrate, the ratio of wild-type cell extract activity PNPP-P/bis-PNP-P being 1·6 after nitrogen starvation but 96 after phosphorus starvation (Table 2). This enables the nitrogen metabolite-repressible enzyme to be monitored without interference from the phosphate-repressible enzyme and allows the relative con-

https://doi.org/10.1017/S0016672300022916 Published online by Cambridge University Press

Table 2. *The responses of intracellular and secreted phosphodiesterase activities to nutrient starvation in strains of various genotypes*

Relevant genotypes	Phosphodiesterase																	
	MM				−P				−N				−C				−S	
	PNPP-P		bis-PNP-P		PNPP-P		bis-PNP-P		PNPP-P		bis-PNP-P		PNPP-P		bis-PNP-P		PNPP-P		bis-PNP-P	
	CE	M	CE	M	CE	M	CE	M	CE	M	CE	M	CE	M	CE	M	CE	M	CE	M
Wild type	< 0·1	< 0·1	< 0·1	< 0·1	3850 ± 160	263 ± 23	40·1 ± 3·1	1·0 ± 0·1	151 ± 12	< 0·1	95·3 ± 2·0	7·2 ± 0·8	83·7 ± 18·2	45·1 ± 9·8	0·4 ± 0·4	< 0·1	7·9 ± 2·5	< 0·1	< 0·1	< 0·1
*palc*A-40	< 0·1	< 0·1	< 0·1	< 0·1	< 0·1	< 0·1	< 0·1	< 0·1	151 ± 17	< 0·1	95·2 ± 2·7	n.d.	73·1 ± 17·0	27·0 ± 5·7	3·2 ± 1·6	< 0·1	4·7 ± 0·4	< 0·1	< 0·1	< 0·1
*are*A^{r}-18	< 0·1	< 0·1	< 0·1	< 0·1	2200 ± 34	293 ± 7	29·2 ± 0·6	0·9 ± 0·3	< 0·1	< 0·1	< 0·1	< 0·1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
*xpr*D-1	143 ± 7	24·0 ± 3·5	82·5 ± 5·7	8·8 ± 1·1	2520 ± 6	198 ± 30	147 ± 33	6·7 ± 1·7	388 ± 21	14·0 ± 2·5	252 ± 17	36·9 ± 6·8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
creA^{d}-1	< 0·1	< 0·1	< 0·1	< 0·1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	78·4 ± 6·7	17·0 ± 1·3	7·5 ± 0·6	< 0·1	n.d	n.d.	n.d.	n.d.

Cell-free extracts (CE) or culture medium (M) were assayed after growth of mycelia for 12 h at 37 °C in appropriately supplemented shaken liquid minimal medium (MM) or additionally followed by 6 h in the same conditions in medium lacking phosphate (−P), a carbon source (−C) or a sulphur source (−S) or by 4 h in the same conditions in medium lacking a nitrogen source (−N). Specific activities towards *p*-nitrophenyl phenylphosphonate (PNPP-P) and *bis*-(*p*-nitrophenyl)-phosphate (bis-PNP-P) are given ± 1 S.D. n.d., Not determined.

tributions of the two enzymes to be assessed in measurements of total phosphodiesterase activity. The phosphate-repressible enzyme does, however, catalyse the hydrolysis of *p*-nitrophenyl phenylphosphonate (PNPP-P), a convenient substrate for enzyme assays, and *β*-naphthyl phenylphosphonate, a convenient substrate for staining both colonies and gels in the presence of a suitable diazonium salt.

The inability of bis-PNP-P to serve as a significant substrate for the phosphate-repressible phosphodiesterase enables data in Table 2 to confirm that the *are*A gene product mediates nitrogen metabolite repression of the phosphodiesterase-phosphomonoesterase. The loss-of-function mutation *are*A^{r}-18 completely eliminates any response of this enzyme to nitrogen starvation whereas the *are*A allele *xpr*D-1, leading to nitrogen metabolite derepression, considerably elevates both intra- and extracellular levels of this enzyme under both nitrogen sufficiency and nitrogen starvation. These effects are also apparent in measurements of acid phosphomonoesterase levels (Table 3).

The second convenient feature of phosphate-repressible phosphodiesterase is its extraordinary degree of phosphate-repressibility (Table 2). Upon phosphorus starvation of the wild type, intracellular levels increase by a factor of more than 38000 whilst extracellular levels increase more than 260-fold. It is thus an exquisitely sensitive indicator of phosphate repression.

(iii) *Phosphate repression is mediated by the* palc*A gene product*

The *palc*A gene was identifed by Dorn (1965*a*, *b*) as a gene where mutations lead to loss of both acid and alkaline phosphatases. The electrophoresis pattern in Fig. 1 shows that the *palc*A-40 mutation abolishes phosphate derepression of alkaline phosphatase PI and acid phosphatases PIII and PV. Data in Table 1 confirm this result for PI. In a strain carrying *pal*G-21 to eliminate alkaline phosphatase PII, *palc*A-40 abolishes phosphate derepression of alkaline phosphatase. Data in Table 3 show that *palc*A-40 completely eliminates the response of phosphodiesterase to phosphate starvation. For intracellular activity towards PNPP-P, *palc*A-40 exerts an effect of more that 38000-fold. Data in Table 4 show that *palc*A-40 also abolishes phosphate derepression of acid phosphatase. A strain carrying *pac*A-1, a structural gene mutation leading to loss of PV (Caddick & Arst, 1986) has been included to aid assessment of the relative contributions of PV to the total acid phosphatase levels. Assays at pH 6 monitor mainly PV (Harsanyi & Dorn, 1972; Caddick & Arst, 1986) and show that *palc*A-40 prevents phosphate derepression of this enzyme whereas the pH 4·6 data indicate a similar effect on one or more other enzymes, presumably including PIII. In contrast, *palc*A-40 does not affect responses to carbon, nitrogen or sulphur starvation (Fig. 1; Tables 1, 3 and 4).

https://doi.org/10.1017/S0016672300022916 Published online by Cambridge University Press

Table 3. *The responses of intracellular and secreted acid phosphatase activities to nutrient starvation in strains of various genotypes*

Relevant genotype	Acid phosphatase													
	MM			−P			−N			−C			−S	
	pH 6		pH 4·6	pH 6		pH 4·6	pH 6		pH 4·6	pH 6		pH 4·6	pH 6		pH 5·6
	CE	M	CE	CE	M	CE	CE	M	CE	CE	M	CE	CE	M	CE
Wild type	7·1±0·9	< 0·1	13·8±1·3	338±26	30·7±4·1	118±11	59·8±7·0	0·3±0·0	174±19	111±20	17·8±0·4	53·1±5·5	10·2±2·9	< 0·1	28·0±1·6
pacA-1	6·8±0·2	< 0·1	16·0±0·4	43·4±3·6	1·9±0·3	49·8±4·2	54·6±3·7	0·3±0·0	163±6	92·7±1·7	13·2±0·8	51·3±3·4	10·4±0·3	< 0·1	27·2±0·7
palcA-40	7·9±0·5	< 0·1	14·1±1·1	12·6±1·6	0·3±0·0	21·4±1·5	50·0±12·4	0·3±0·0	162±25	105±3	17·8±0·0	50·2±1·6	8·6±1·4	< 0·1	27·3±3·5
areA^{r}-18	7·6±0·9	< 0·1	12·0±1·3	339±27	n.d.	137±16	8·4±3·4	< 0·1	13·6±3·7	57·9±3·3	2·8±1·1	30·4±2·5	6·3±0·5	< 0·1	23·5±3·3
xprD-1	38·5±1·0	4·9±0·6	74·2±1·1	202±13	20±1·1	284±17	125±6	2·7±0·1	466±17	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
creA^{d}-1	7·0±0·7	< 0·1	14·3±0·8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	104±11	5·7±0·3	51·3±6·8	n.d.	n.d.	n.d.

Cell-free extracts (CE) or culture media (M) were assayed after growth of mycelia for 12 h at 37 °C in appropriately supplemented shaken liquid minimal medium (MM) or additionally followed by 6 h in the same conditions in medium lacking phosphate (−P), a carbon source (−C) or a sulphur source (−S) or by 4 h in the same conditions in medium lacking a nitrogen source (−N). Specific activities for assays at pH 6 in the presence of 2 mM-EDTA and at pH 4·6 in the presence of 10 mM $MgCl_2$ are given ±1 S.D. n.d., Not determined.

*palc*A probably also controls the expression of at least one phosphate permease. *palc*A mutations confer resistance to the toxic phosphate analogue arsenate and a number of *palc*A mutations have been selected as resulting in resistance to 20 mM arsenate in complete medium (Caddick, unpublished results). A further indication that *palc*A mutations reduce phosphate uptake is that they result in an elevated phosphate requirement for growth. Whereas wild-type strains are able to form thin colonies lacking aerial hyphae but of nearly normal diameter on $-P_i$ medium, strains carrying a more extreme *palc*A mutation such as *palc*A-40 do not grow at all on $-P_i$ medium. If $-P_i$ medium is made 100 μM with respect to phosphate, growth of *palc*A-40 and wild-type strains becomes equivalent.

*palc*A mutations are fully recessive to the wild-type allele in diploids, as judged both by colony staining tests and by enzyme assays (data not shown). *palc*A mutations of the existing class (e.g. *palc*A-1 and -40) can be easily selected without the aid of a powerful positive selection technique, for example by colony staining (Dorn, 1965*a*, Caddick & Brownlee, unpublished results). There is therefore no reason to suppose that they result only from rare events. In view of this and of their recessivity, *palc*A-40 and other existing *palc*A mutant alleles can be reasonably classified as loss-of-function mutations and therefore *palc*A can be considered positive-acting.

In heterokaryons *palc*A-40 fails to complement structural gene mutations leading to loss of two of the activities under *palc*A control, PI and PV (Table 4). This might indicate nuclear limitation of the *palc*A product or more mundanely arise from its presence in limiting concentrations, but in either case it provides additional evidence that expression of both *pac*A and *pal*D is under *palc*A control (see Scazzocchio & Arst, 1978 and references therein).

The map position of *palc*A in the centromere-proximal region of the left arm of linkage group II has been reported previously (Arst, Rand & Bailey, 1979; Arst & Bailey, 1980).

(iv) *Mutations in* pac*J probably reduce phosphate uptake at acidic pH*

*pac*J mutations were selected as conferring arsenate resistance at low pH (see Materials and Methods section). They do not confer arsenate resistance at high growth pH (e.g. pH 8). A similar pH effect can be seen on phosphatase regulation. *pac*J mutations considerably enhance colony staining for acid phosphatase and phosphodiesterase in phosphate-sufficient (i.e. minimal) medium at a growth pH of 5 or, to a lesser extent, 6·5 but not 8. No such effect is seen with alkaline phosphatase.

These phosphatase colony staining results are confirmed by enzyme measurements shown in Tables 5 and 6. The first point to note in these Tables is that in

https://doi.org/10.1017/S0016672300022916 Published online by Cambridge University Press

Table 4. *Heterokaryon complementation analysis of a* palcA^- *mutation with* pacA^- *and* palD^- *mutations*

Relevant genotype of heterokaryon	Alkaline phosphatase	Acid phosphatase	Phospho-diesterase
+/+	+	+	+
*palc*A-40/ +	+	+	+
*pal*D-8/ +	+	+	+
*pac*A-1/ +	+	+	+
*palc*A-40/*pal*D-8	−	+	+
*palc*A-40/*pac*A-1	+	−	+

The *palc*A-40 strain also carried *w*A-4 (white conidial colour) and *paba*A-1 (*p*-amino-benzoate requirement). The *pal*D-8 and *pac*A-1 strains also carried *y*A-2 (yellow conidial colour) and *panto*B-100 (D-pantothenate requirement). The wild-type strain used carried either *paba*A-1 or *y*A-2 and *panto*B-100, chosen so as to complement the other component strain. Balanced, strongly growing heterokaryons maintained on unsupplemented solid minimal medium containing (final concentration) 10 mM nitrate as nitrogen source at 37 °C were transferred to unsupplemented solid $-P_i$ medium containing (final concentration) 10 mM ammonium as nitrogen source and grown for a further 2–3 days at 37 °C and stained with the appropriate colony staining solution. In each case multiple transfers were tested.

Table 5. *The effects of a* pacJ^- *mutation on acid phosphatase activities with and without phosphate starvation in media differing in pH*

pH of growth medium	Relevant genotype	Acid phosphatase			
		$+P_i$		$-P_i$	
		pH 6	pH 4·6	pH 6	pH 4·6
5	*pac*J$^+$	7·5±1·0	14·9±0·8	507±5	238±14
5	*pac*J-121	113±4	76·3±2·8	592±54	292±32
6·5	*pac*J$^+$	7·1±0·9	13·8±1·3	338±26	118±11
6·5	*pac*J-121	19·6±1·8	22·2±1·2	348±14	120±2
8	*pac*J$^+$	5·3±0·4	9·9±1·1	230±16	70·0±12·1
8	*pac*J-121	5·0±1·2	9·7±0·7	294±12	87·9±8·4

Cell-free extracts were prepared from mycelia grown for 12 h at 37 °C in appropriately supplemented shaken liquid minimal medium ($+P_i$) buffered at pH 5, 6·5 or 8 or from mycelia supplemented shaken liquid minimal medium at pH 6·5 followed by 6 h in the same conditions but in medium lacking phosphate ($-P_i$) buffered at pH 5, 6·5 or 8. Specific activities for assays at pH 6 in the presence of 2 mM-EDTA or at pH 4·6 in the presence of 10 mM-$MgCl_2$ are given ±1 S.D.

Table 6. *The effects of a* pacJ^- *mutation on phosphodiesterase activities with and without phosphate starvation in media differing in pH*

pH of growth medium	Relevant genotype	Phosphodiesterase			
		$+P_i$		$-P_i$	
		PNPP-P	bis-PNP-P	PNPP-P	bis-PNP-P
5	*pac*J$^+$	< 0·1	< 0·1	4150±120	61·0±1·9
5	*pac*J-121	508±86	1·7±0·1	5390±300	79·2±11·4
6·5	*pac*J$^+$	< 0·1	< 0·1	3580±160	40·1±3·1
6·5	*pac*J-121	13·6±6·3	< 0·1	3610±250	44·0±1·2
8	*pac*J$^+$	< 0·1	< 0·1	1720±120	26·7±0·9
8	*pac*J-121	< 0·1	< 0·1	1600±190	30·6±2·3

Cell-free extracts were prepared from mycelia growth for 12 h at 37 °C in appropriately supplemented shaken liquid minimal medium ($+P_i$) buffered at pH 5, 6·5 or 8 or from mycelia grown for 12 h at 37 °C in appropriately supplemented shaken liquid minimal medium at pH 6·5 followed by 6 h in the same conditions but in medium lacking phosphate ($-P_i$) buffered at pH 5, 6·5 or 8. Specific activities towards *p*-nitrophenyl phenylphosphonate (PNPP-P) and *bis*-(*p*-nitrophenyl)-phosphate (bis-PNP-P) are given ±1 S.D.

https://doi.org/10.1017/S0016672300022916 Published online by Cambridge University Press

strains wild-type for phosphate-repressible enzymes the levels of acid phosphatase and phosphodiesterase increase as the pH is lowered. The reverse is true for alkaline phosphatase (data not shown). The regulation of expression of genes specifying phosphatases and other activities by the pH of the growth medium will be the subject of a separate report (Caddick, Brownlee & Arst, in preparation). For present purposes it is important to note that comparisons between strains must be done at the same growth pH. Data in Table 5 show that in phosphate-sufficient (i.e. minimal) medium *pac*J-121 results in appreciable phosphate derepression if acid phosphatase activity is measured at a growth pH of 5 but not 8. The effect is more pronounced when assays are done at pH 6 in the presence of EDTA than when they are done at pH 4·6 in the presence of Mg^{2+}. This indicates that the major effect is on PV levels (Caddick & Arst, 1986), a conclusion confirmed by colony staining tests: *pac*A-1 *pac*J-121 double mutants are indistinguishable from *pac*A-1 single mutants with respect to acid phosphatase staining after growth at 37 °C although they retain other aspects of the *pac*J-121 phenotype. Table 6 shows that *pac*J-121 exerts a similar growth pH-dependent effect on phosphodiesterase levels. As this effect is much more pronounced when PNPP-P rather than bis-PNP-P serves as substrate, it is clearly exerted on the phosphate-repressible rather than the nitrogen metabolite-repressible enzyme (see Section (ii) of Results and Discussion). Data not shown confirm that *pac*J-121 has no effect on alkaline phosphatase levels. Whether PI and PII are monitored together using *pal*G$^+$ *pal*D$^+$ strains or individually using *pal*G-21 (to eliminate PII) or *pal*D-8 (to eliminate PI) strains, *pac*J-121 and *pac*J$^+$ strains do not differ significantly. The lack of effect of *pac*J mutations on alkaline phosphatase levels might be associated with the fact that they exert their regulatory effect at acidic growth pH whereas phosphate-repressible alkaline phosphatase (PI) is synthesized optimally at alkaline growth pH (Caddick *et al.*, in preparation).

The growth pH-dependent arsenate resistance and phosphate derepression resulting from *pac*J mutations suggest that *pac*J might specify a permease which plays an important role in phosphate (and arsenate) uptake at low but not high pH (i.e. a permease for $H_2PO_4^-$). Although direct uptake measurements to test this hypothesis would obviously be desirable (but lie outside the mainstream of our interests in the regulation of phosphatase structural gene expression), the properties of *pac*J-121 *palc*A-1 double mutants do lend some support. These double mutants are unable to grow on solid minimal medium buffered at pH 5. On solid minimal medium buffered at pH 6·5 they grow poorly but they grow normally if the buffering is at pH 8. This would be a likely phenotype if *pac*J mutations affect a different phosphate permease from that under *palc*A control and if the two permeases are wholly responsible for phosphate uptake at acid pH. With respect to phosphatase colony staining phenotype, *palc*A-1 is epistatic to *pac*J-121, showing that the involvement of *pac*J is less direct than that of *palc*A.

Haploidization analysis located *pac*J-121 to linkage group I. Meiotic analysis shows that it is on the left arm tightly linked (< 1 cM) to *an*A, probably on the centromere-distal side, between *an*A and *ribo*A. (Definitions of gene symbols are given by Clutterbuck (1984).)

(v) r *mutations probably have no effect on phosphatase activity or regulation*

Dorn (1965*a*) selected three mutations designated *r*A-1, -2 and -3 as leading to enhanced colony staining for alkaline phosphatase on $-P_i$ medium. These mutations, which pleiotropically reduce conidiation, define a gene located 18 cM centromere-distal to *gal*D on the left arm of linkage group I (Dorn, 1967). Subsequently, Dorn & Rivera (1965) described two other loci, *r*B on the right arm of linkage group II and *r*C on the right arm of linkage group VIII (Dorn, 1967) where mutations increase colony staining for acid phosphatase on $-P_i$ medium. Unfortunately these mutations have apparently been lost. We have, however, selected other mutations having a similar phenotype and they tell a cautionary tale.

*r*D-30 and *r*E-40 are cryosensitive mutations apparently leading to elevated colony staining for alkaline phosphatase, phosphodiesterase and, to a lesser extent, acid phosphatase on both minimal (phosphate-sufficient) and $-P_i$ media at 25 °C but not at 42 °C. *r*D-30 strains conidiate poorly at 25 °C and not at all at 37 °C or above. *r*E-40 strains grow and conidiate poorly at all temperatures. However, using both starch gel electrophoresis and enzyme assays no detectable effect of either mutation on phosphatase levels can be seen after growth at 25 °C in phosphate sufficient or starvation conditions (data not shown). This prompted a re-examination of colony staining. Unlike wild-type strains, *r*D-30 and *r*E-40 strains, after growth at 25 °C on minimal or $-P_i$ medium, stain intensely when the phosphatase substrates (α-naphthyl acid phosphate for acid and alkaline phosphatases, β-naphthyl phenylphosphonate for phosphodiesterase) are omitted from the staining mixtures. This indicates that these mutations enable reactions to take place converting the diazonium salts fast violet B, fast garnet GBC and fast red TR to coloured precipitates. Mutations in at least three other loci, including *daf*A (Caddick & Arst, 1986), can result in a similar although possibly less pronounced phenotype.

Genetic analysis indicates that none of the mutations reported here as having an *r* phenotype is likely to be an *r*B or *r*C allele. However, the map position of *r*D-30, located to linkage group I by haploidization and mapped further meiotically (Fig. 3), does suggest that it might be an *r*A allele. *r*E-40 was located to linkage group VII but no meiotic linkage to a number of other linkage group VII markers has been detected.

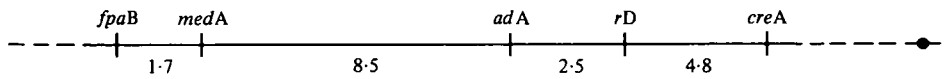

Fig. 3. Map position of *r*D on the left arm of linkage group I. Map positions shown with distances in centimorgans are based on three crosses involving analysis of between 200 and 1959 progeny. Strains used carried various combinations of the mutant alleles *fpa*B-37, *med*A-15, *ad*A-3, *r*D-30 and *cre*A^{d}-1. Selection of certain recombinant progeny was used to confirm the gene order shown above. From a cross of relevant partial genotype *ad*A-3 *cre*A^{d}-1 × *r*D-30, 22 of 24 *ad*A^{+} *r*D^{+} recombinants carry *cre*A^{d}-1 and all 12 *r*D^{+} *cre*A^{+} recombinants carry *ad*A-3. From a cross of relevant partial genotype *fpa*B-37 *med*A-15 *ad*A-3 × *r*D-30, 1 out of 30 *ad*A^{+} *r*D^{+} recombinants is *fpa*B-37 *med*A-15 whereas the other 29 are *fpa*B^{+} *med*A^{+} and 23 out of 27 *med*A^{+} *r*D^{+} progeny carry *ad*A-3 and *fpa*B^{+} whereas the other 4 are *ad*A^{+} *fpa*B^{+}. The position of the centromere (-●-) is from Clutterbuck (1984) where definitions of gene symbols can also be found. The positioning of other markers in this region of the genome, including *gal*D 0·6 cM centromere-proximal to *cre*A, is given by Arst, Brownlee & Cousen (1982) and Wiame, Grenson & Arst (1985).

This work was supported by the Science and Engineering Research Council through a studentship to M.X.C. and a research grant to H.N.A. We thank Shelagh Cousen for technical assistance.

References

Al Taho, N. M., Sealy-Lewis, H. M. & Scazzocchio, C. (1984). Supressible alleles in a wide domain regulatory gene in *Aspergillus nidulans*. *Current Genetics* **8**, 245–251.

Arst, H. N., Jr. (1981). Aspects of the control of gene expression in fungi. *Symposia of the Society for General Microbiology* **31**, 131–160.

Arst, H. N., Jr. (1982). A near terminal pericentric inversion leads to nitrogen metabolite derepression in *Aspergillus nidulans*. *Molecular and General Genetics* **188**, 490–493.

Arst, H. N., Jr. (1984). Regulation of gene expression in *Aspergillus nidulans*. *Microbiological Sciences* **1**, 137–141.

Arst, H. N., Jr. & Bailey, C. R. (1977). The regulation of carbon metabolism in *Aspergillus nidulans*. In *Genetics and Physiology of Aspergillus* (ed. J. E. Smith and J. A. Pateman), pp. 131–146. London: Academic Press.

Arst, H. N., Jr. & Bailey, C. R. (1980). Genetic evidence for a second asparaginase in *Aspergillus nidulans*. *Journal of General Microbiology* **121**, 243–247.

Arst, H. N., Jr. Brownlee, A. G. & Cousen, S. A. (1982). Nitrogen metabolite repression in *Aspergillus nidulans*: a farewell to *tam*A? *Current Genetics* **6**, 245–257.

Arst, H. N., Jr. & Cove, D. J. (1969). Methylammonium resistance in *Aspergillus nidulans*. *Journal of Bacteriology* **98**, 1284–1293.

Arst, H. N., Jr. & Cove, D. J. (1973). Nitrogen metabolite repression in *Aspergillus nidulans*. *Molecular and General Genetics* **126**, 111–141.

Arst, H. N., Jr., Rand, K. N. & Bailey, C. R. (1979). Do the tightly linked structural genes for nitrate and nitrite reductases in *Aspergillus nidulans* form an operon? Evidence from an insertional translocation which separates them. *Molecular and General Genetics* **174**, 89–100.

Arst, H. N., Jr. & Scazzocchio, C. (1985). Formal genetics and molecular biology of the control of gene expression in *Aspergillus nidulans*. In *Gene Manipulations in Fungi*, Chapter 13 (ed. J. W. Bennett and L. L. Lasure), pp. 309–343. New York: Academic Press.

Arst, H. N., Jr., Tollervey, D. W. & Sealy-Lewis, H. M. (1982). A possible regulatory gene for the molybdenum-containing cofactor in *Aspergillus nidulans*. *Journal of General Microbiology* **128**, 1083–1093.

Bailey, C. & Arst, H. N., Jr. (1975). Carbon catabolite repression in *Aspergillus nidulans*. *European Journal of Biochemistry* **51**, 573–577.

Bal, J., Kajtaniak, E. M. & Pieniazek, N. J. (1977). 4-nitroquinoline-1-oxide: a good mutagen for *Aspergillus nidulans*. *Mutation Research* **56**, 153–156.

Brownlee, A. G. & Arst, H. N. Jr. (1983). Nitrate uptake in *Aspergillus nidulans* and involvement of the third gene of the nitrate assimilation gene cluster. *Journal of Bacteriology* **155**, 138–1146.

Brownlee, A. G., Caddick, M. X. & Arst, H. N., Jr. (1983). A novel phosphate-repressible phosphodiesterase in *Aspergillus nidulans*. *Heredity* **51**, 529.

Caddick, M. X. & Arst, H. N., Jr. (1986). Structural genes for phosphatases in *Aspergillus nidulans*. *Genetical Research* **47**, 83–91.

Clutterbuck, A. J. (1974). *Aspergillus nidulans*. In *Handbook of Genetics*, vol. 1 (ed. R. C. King), pp. 447–510. New York: Plenum Press.

Clutterbuck, A. J. (1984). Loci and linkage map of the filamentous fungus *Aspergillus nidulans*. (Eidam) Winter ($n = 8$). *Genetic Maps* **3**, 265–273.

Cohen, B. L. (1972). Ammonium repression of extracellular protease in *Aspergillus nidulans*. *Journal of General Microbiology* **71**, 293–299.

Cove, D. J. (1966). The induction and repression of nitrate reductase in the fungus *Aspergillus nidulans*. *Biochimica et Biophysica Acta* **113**, 51–56.

Cove, D. J. (1976). Chlorate toxicity in *Aspergillus nidulans*. Studies of mutants altered in nitrate assimilation. *Molecular and General Genetics* **146**, 147–159.

Dorn, G. (1965*a*). Genetic analysis of the phosphatases in *Aspergillus nidulans*. *Genetical Research* **6**, 13–26.

Dorn, G. (1965*b*). Phosphatase mutants in *Aspergillus nidulans*. *Science* **150**, 1183–1184.

Dorn, G. L. (1967). A revised map of the eight linkage groups of *Aspergillus nidulans*. *Genetics* **56**, 619–631.

Dorn, G. & Rivera, W. (1965). Supplementary list of located or partially located mutants in *A. nidulans*. *Aspergillus Newsletter* **6**, 13–15.

Harsanyi, Z. & Dorn, G. L. (1972). Purification and characterization of acid phosphatase V from *Aspergillus nidulans*. *Journal of Bacteriology* **110**, 246–255.

Hynes, M. J. (1975). Studies on the role of the *are*A gene in the regulation of nitrogen catabolism in *Aspergillus nidulans*. *Australian Journal of Biological Sciences* **28**, 301–313.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.

McCully, K. S. & Forbes, E. (1965). The use of *p*-fluorophenylalanine with 'master strains' of *Aspergillus nidulans* for assigning genes to linkage groups. *Genetical Research* **6**, 352–359.

Polya, G. M., Brownlee, A. G. & Hynes, M. J. (1975). Enzymology and genetic regulation of a cyclic nucleotide-binding phosphodiesterase-phosphomonoesterase from *Aspergillus nidulans*. *Journal of Bacteriology* **124**, 693–703.

Pontecorvo, G., Roper, J. A., Hemmons, L. M., Macdonald, K. D. & Bufton, A. W. J. (1953). The genetics of *Aspergillus nidulans*. *Advances in Genetics* **5**, 141–238.

Rand, K. N. & Arst, H. N. Jr. (1977). A mutation in *Aspergillus nidulans* which affects the regulation of nitrite reductase and is tightly linked to its structural gene. *Molecular and General Genetics* **155**, 67–75.

Scazzocchio, C. & Arst, H. N., Jr. (1978). The nature of an initiator constitutive mutation in *Aspergillus nidulans*. *Nature* **274**, 177–179.

Tollervey, D. W. & Arst, H. N., Jr. (1981). Mutations to constitutivity and derepression are separate and separable in a regulatory gene of *Aspergillus nidulans*. *Current Genetics* **4**, 63–68.

Wiame, J.-M., Grenson, M. & Arst, H. N., Jr. (1985). Nitrogen catabolite repression in yeasts and filamentous fungi. *Advances in Microbial Physiology* **26**, 1–88.

The work cited as 'in preparation' is now in press in *Molecular and General Genetics*: 'Regulation of gene expression by pH of the growth medium in *Aspergillus nidulans*' by M. X. Caddick, A. G. Brownlee & H. N. Arst, Jr.