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Structural genes for phosphatases in *Aspergillus nidulans*

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Summary

Although the fungus *Aspergillus nidulans* has a multiplicity of phosphatases and of genes where mutations affect one or more phosphatases, we have succeeded in identifying structural genes for three phosphatases as well as one other gene which might encode a fourth. Using both conditional and non-conditional mutations, *pal*D has been shown to be the structural gene for a phosphate-repressible alkaline phosphatase, *pal*G to be the structural gene for a non-repressible alkaline phosphatase which apparently exists in two electrophoretically distinct forms (but whose rates of thermal inactivation are apparently very similar) and *pac*A to be the structural gene for both intracellular and secreted forms of a phosphate-repressible acid phosphatase. Colony staining techniques for the enzymes specified by *pal*D and *pac*A have been described previously but we have now shown that the enzyme specified by *pal*G can be detected by staining toluene-permeabilized colonies. Mutations in *pac*G lead to loss of non-repressible acid phosphatase as judged by colony staining and electrophoretic patterns but their effects on assays of activity in cell-free extracts are only marginal. Under phosphate-limited, but not phosphate-starved or phosphate-sufficient, conditions, *pac*G$^-$ mutations also affect the regulation of other, phosphate-repressible phosphatases. None of these phosphatases, alone or in combination, plays an essential role.

1. Introduction

Like many organisms, the ascomycete fungus *Aspergillus nidulans* has a multiplicity of phosphatases (Dorn, 1965*a*, *b*, 1967, 1968; Dorn & Rivera, 1966; Harsanyi & Dorn, 1972; Polya, Brownlee & Hynes, 1975; Brownlee, Caddick & Arst, 1983). Dorn (1965*a*, *b*) identified fifteen loci where mutations apparently affect one or more phosphatases. In no case, however, could a precise gene role be defined and the large number of genes involved presented a bewildering complexity. Nevertheless, the phosphatases of *A. nidulans* are worthy of study because their syntheses are subject to diverse and particularly interesting forms of regulation.

A prerequisite to the study of phosphatase regulation is to define conditions for examining the synthesis of single enzymes. Ideally this consists of identification of the structural gene(s) specifying the enzyme plus a suitable genetic background and growth and assay conditions for monitoring the enzyme without complications caused by the presence of other enzymes having similar activities. Here we report identification of structural genes for one acid and two alkaline phosphomonesterases and define conditions in which each of these enzymes can be efficiently monitored *in vivo* and *in vitro*. We also identify a putative structural gene for another acid phosphatase.

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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 minimal medium of Cove (1966) containing (final concentrations) 1% (w/v) D-glucose as carbon source and, usually, 10 mM ammonium (as the (+)-tartrate) as nitrogen source was used. Phosphate-free medium ($-P_i$ medium) was prepared by substituting chloride salts for phosphate salts at equimolar cation concentrations. 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) with the exceptions listed below. *pal*D-100, -103, -105, -106 and -111 were selected after 4-nitroquinoline-1-oxide (NQO) mutagenesis (Bal, Kaj-

taniak & Pieniazek, 1977) of a strain of genotype *paba*A-1 (*p*-amino-benzoate – requiring) as failing to stain for alkaline phosphatase by the method of Dorn (1965*a*) after growth on $-P_i$ medium supplemented with 100 μM phosphate (P_i) and buffered at pH $\sim$ 8 with 10 mM tris HCl. *pal*D-100 and -106 appear to be recessive in heterozygous diploids in staining tests and fail to complement *pal*D-8 in a diploid. The other three mutations were not tested for dominance, but in heteroallelic diploids with *pal*D-8, no alkaline phosphatase is detectable by the colony staining technique of Dorn (1965*a*). All five segregate as single mutations in crosses and are tightly linked to *pal*D-8 ($< 0{\cdot}1$ cM in the case of *pal*D-106). *pal*D-105, -106, and -111 are thermosensitive, leading to staining for alkaline phosphatase after growth at 25 °C but not at 37 °C.

*pal*G-21, -22, -23 and -24 were selected after NQO mutagenesis of a strain of genotype *paba*A-1 as failing to stain for alkaline phosphatase after growth on minimal (high phosphate) medium for 2 days at 42 °C followed by toluene-permeabilization (Scazzocchio, Sdrin & Ong, 1982). (Selection was carried out after growth at 42 °C in order to maximise the proportion of thermosensitive mutations.) All four mutations are codominant in heterozygous diploids, and diploids heteroallelic for *pal*G-21 and *pal*G-22, -23 or -24 fail to stain for alkaline phosphatase after growth on high phosphate medium and toluene-permeabilization. All four segregate as single mutations in crosses, and *pal*G-22, -23 and -24 are all tightly linked ($< 0{\cdot}3$ cM) to *pal*G-21. They therefore define a heretofore unidentified gene, *pal*G. *pal*G-22, -23 and -24 are thermosensitive, leading to staining after growth at 25 °C but not at 37 °C (or 42 °C).

*pac*A-100 and -101 were selected after NQO mutagenesis of a strain of genotype *y*A-2 (yellow conidial colour) *panto*B-100 (D-pantothenate-requiring) *pal*D-8 (lacking repressible alkaline phosphatase) as failing to stain for acid phosphatase using the method of Dorn (1965*a*) except that staining was carried out in 0·3 M maleate (as the Na^+ salt) buffer at pH 6·5 with fast red TR as the diazonium salt. Both mutations are codominant in heterozygous diploids, and diploids heteroallelic for *pac*A-1 and *pac*A-100 or -101 fail to stain for acid phosphatase on $-P_i$ medium. Both segregate as single mutations in crosses and are tightly linked ($< 0{\cdot}14$ cM) to *pac*A-1. They can therefore be classified as *pac*A alleles. *pac*A-1 and -100 are thermosensitive, leading to staining after growth at 25 °C but not 37 °C. Using haploidization analysis (McCully & Forbes, 1965), it was shown that a *pac*A-101 strain carries a translocation involving linkage groups IV (containing *pac*A) and VII. It is therefore possible that this non-leaky and non-conditional allele results from a translocation breakpoint in the *pac*A gene.

*pac*G-91 and -110 were selected after ultraviolet mutagenesis and *pac*G-92 after NQO mutagenesis of a strain of genotype *paba*A-1 as resulting in lack of staining for acid phosphatase by the method of Dorn (1965*a*) after growth on $-P_i$ medium supplemented with 100 μM P_i. All three mutations appear condominant with *pac*G^+ in diploids in plate tests, and *pac*G-92 and -110 fail apparently to complement *pac*G-91 in a heteroallelic diploid. All three segregate as single mutations in crosses and *pac*G-92 and -110 are tightly linked to *pac*G-91 (< 1 cM). They are therefore likely to be allelic. *pac*G-91 is a leaky, thermosensitive mutation whose phenotype is most extreme at 42 °C, the growth temperature at which it was selected. *pac*G-92 and -110 were selected after growth at 37 °C.

(ii) *Colony staining*

Detection of acid and alkaline phosphatases in colonies followed the method of Dorn (1965*a*) except that, for acid phosphatase, fast red TR salt, at equal w/v, replaced fast garnet GBC salt. (Staining with fast red is faster and gives greater contrast). Phosphate-repressible phosphodiesterase was detected by staining colonies grown on $-P_i$ medium with 0·6 M acetate (as the Na^+ salt) buffer at pH 4·8 containing the substrate β-naphthyl phenylphosphonic acid and the diazonium salt fast garnet GBC at 500 μg/ml and 5 mg/ml, respectively.

(iii) *Starch gel electrophoresis*

Starch gel electrophoresis was carried out by the method of Dorn (1965*a*) using 13% (w/v) starch except that the gels contained 7% (w/v) urea. Gels were stained by flooding with the appropriate colony staining solution for approximately 45 min at room temperature.

(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, usually, 20 mM ammonium (as the (+)-tartrate) as the nitrogen source was used. $-P_i$ liquid medium was prepared by substituting chloride salts for phosphate salts at equimolar cation concentrations. All strains used for enzyme assays or starch gel electrophoresis carry the *p*-aminobenzoate auxotrophy *paba*A-1, and 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*

Mycelia ($\sim$300 mg wet weight) were ground with an equal weight of acid-washed sand in a chilled mortar for several minutes in $2 \times 2{\cdot}5$ ml of extraction buffer (20 mM tris-HCl, pH 8, containing 2 mM-EDTA, 1 mM benzamidine HCl and 4·3 mM 2-mercaptoethanol),

half of which was added halfway through grinding, at 4 °C. The crude homogenate was centrifuged at 24000 *g* for 15 min at 4 °C and the supernatant taken for enzyme assay. An identical procedure was followed for preparing extracts for starch gel electrophoresis except that the second 2·5 ml aliquot of extraction buffer was not added, to give extracts two-fold more concentrated.

For enzyme assays 20 μl of cell-extract or 50 μl of culture medium was made to 1 ml using the appropriate buffer containing 1 mM *p*-nitrophenylphosphate (disodium salt) as substrate. Acid phosphatase (EC 3.1.3.2) was assayed (as indicated) in 100 mM acetate (sodium salt) buffer at pH 4·6 containing 10 mM-$MgCl_2$ or in 100 mM maleate (sodium salt) buffer at pH 6 containing 2 mM-EDTA. Alkaline phosphatase (EC 3.1.3.1) was assayed in 100 mM diethanolamine (hydrochloride) buffer at (as indicated) pH 9·5 or 10, both containing 10 mM-$MgCl_2$. All phosphatase reactions were terminated by 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.

For enzyme thermolability studies, the time course of activity was also measured in equal activity mixtures of extracts from the strains being compared. The demonstration that such a mixture gave a thermolability curve intermediate between those of the two strains measured separately was used to conclude that the differences observed were inherent properties of the phosphatases under study and not due to some other substance present (in excess) in an extract.

3. Results and Discussion

(i) pal*D is a structural gene for the phosphate-repressible alkaline phosphomonoesterase PI*

Dorn (1965*a*, *b*, 1967, 1968) partially purified and characterised two alkaline phosphatases designated PI and PII and showed that the non-pleiotropic mutation *pal*D-8, selected as resulting in lack of alkaline phosphatase staining on medium lacking phosphate ($-P_i$ medium), leads to loss of the phosphate-repressible activity PI. The electrophoresis pattern in Fig. 1 confirms this result: under phosphate-derepressing growth conditions, active PI is absent from cell-extracts of *pal*D-8 strains (lanes 1 and 2) but present in *pal*D$^+$ strains (lanes 3 and 4). Data in Table 1 show that under phosphate-derepressing growth conditions *pal*D-8 leads to a two-fold reduction in total alkaline phosphatase activity whilst not affecting levels under repressing conditions or acid phosphatase activity.

Using *pal*G-21 strains to eliminate PII and PII′ (*vide infra*), data in Fig. 2 show that the thermosensitive allele *pal*D-106 leads to decreased thermostability of PI. Using the linear portion of the curves, the half-life at 60 °C of PI from the *pal*D-106 strain can be estimated at 66 min as compared to 136 min for the wild type. Data in Table 2 demonstrate codominance of *pal*D-8 with the wild type (*pal*D$^+$) allele.

The fact that *pal*D-8 leads to loss of PI whilst *pal*D-106 leads to a physically altered enzyme and the lack of pleiotrophy of *pal*D mutations strongly imply that *pal*D is a structural gene for PI. Further meiotic localisation of *pal*D is shown in Fig. 3.

(ii) pal*G is a structural gene for the non-repressible alkaline phosphomonesterases PII and PII′*

Neither mutations affecting non-repressible alkaline phosphatase activity nor any means of detecting the presence (or absence) of this activity in plate tests have been reported previously. On phosphate-sufficient (e.g. minimal) media, no alkaline phosphatase staining using the method Dorn (1965*a*) can be detected in *A. nidulans* colonies unless they are first toluene-permeabilized by the method of Scazzocchio *et al.* (1982). This suggests that the non-repressible alkaline phosphatase is exclusively intracellular. Toluene-permeabilization enabled the selection of *pal*G$^-$ mutations (see Materials and Methods). Although they prevent alkaline phosphatase staining on phosphate-sufficient medium they do not do so on $-P_i$ medium nor do they affect acid phosphatase staining under any growth conditions. Apart from leading to a rather slight reduction in conidiation, they seem to be without any pleiotropic effects. Starch gel electrophoresis shows that *pal*G-21 results in loss of alkaline phosphatase PII as well as of another alkaline phosphatase designated PII′ (Fig. 1). PII′ is not apparent in the gels of Dorn (1965*a*, *b*) because it only enters the gel when extracts are prepared in the presence of a nonionic detergent such as Nonidet P-40, a procedure based on the experience of Nagy *et al.* (1981) with *Chlamydomonas reinhardii* acid phosphatase. A *pal*D-8 *pal*G-21 double mutant lacks PI, PII and PII′ (Fig. 1). Data in Table 1 show that under phosphate-sufficient growth conditions, *pal*G-21 leads to nearly total loss of alkaline phosphatase activity whilst resulting in a two-fold reduction under phosphate-derepressing conditions. Consistent with the electrophoresis patterns, *pal*G-21 and *pal*D-8 are additive in their effects on alkaline phosphatase levels in double mutants (Table 1). *pal*G-21 does not affect acid phosphatase levels.

Using *pal*D-8 strains to eliminate PI (*vide supra*), data in Fig. 4 show that the thermosensitive allele *pal*G-23 leads to decreased thermostability of PII and PII′. The thermal inactivation curves for both *pal*G$^+$ and *pal*G-23 strains follow first-order kinetics. Assuming that PII and PII′ both contribute significant activity under the assay conditions, this indicates that the two forms are equally thermolabile, with half-lives at 40 °C of 6·5 min in the *pal*G-23 strain as compared to 22·5 min for the *pal*G$^+$ strain. Data in

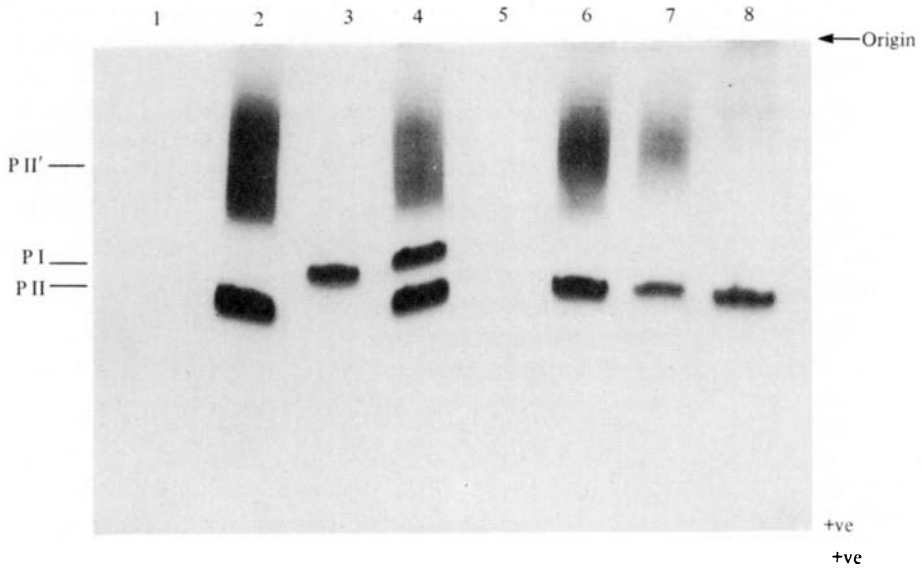

Fig. 1. Effects of *pal*D$^-$ and *pal*G$^-$ mutations on starch gel electrophoretic patterns of alkaline phosphatases. Mycelia were grown in appropriately supplemented shaken liquid minimal medium containing 20 mM (final concentration) ammonium as nitrogen source for 12 h at 37 °C (lanes 7 and 8) or additionally followed by 4 h in the same conditions but lacking a nitrogen source (lanes 5 and 6) or by 6 h in the same conditions but in phosphate-free medium (lanes 1–4). Cell-free extracts for lanes 1–7 were prepared in the presence of 2% (w/v) Nonidet P-40. Lane 1, *pal*D-8 *pal*G-21 strain; lane 2, *pal*D-8 strain; lanes 3 and 5, *pal*G-21 strain; lanes 4, 6, 7 and 8, wild type (*pal*D$^+$ *pal*G$^+$) strain. The gel was stained for alkaline phosphatase activity only.

Table 1. *Effects of* palD$^-$ *and* palG$^-$ *mutations on alkaline and acid phosphatases*

	Alkaline phosphatase		Acid phosphatase	
Relevant genotype	$+P_i$	$-P_i$	$+P_i$	$-P_i$
Wild type (*pal*D$^+$ *pal*G$^+$)	14·2±0·6	85·6±6·2	7·1±0·9	338±26
*pal*D-8 *pal*G$^+$	13·9±0·4	45·3±9·1	7·5±0·7	329±44
*pal*D$^+$ *pal*G-21	0·8±0·1	47·9±8·2	6·7±0·5	315±14
*pal*D-8 *pal*G-21	0·1±0·1	< 0·1	6·5±0·3	321±3

Cell-free extracts were prepared from mycelia grown for 12 h at 37 °C in appropriately supplemented shaken liquid minimal medium containing 20 mM (final concentration) ammonium as nitrogen source ($+P_i$) or additionally followed by 6 h in the same conditions but in phosphate-free medium ($-P_i$). Alkaline phosphatase activity was assayed in 100 mM diethanolamine buffer at pH 10. Acid phosphatase activity was assayed in 100 mM maleate (as the Na^+ salt) buffer at pH 6 in the presence of 2 mM-EDTA. Specific activities (±1 S.D.) are expressed in nmoles *p*-nitrophenol liberated per mg soluble protein in extract per minute at 30 °C.

Table 2 show that *pal*G-21 is codominant with the wild type (*pal*G$^+$) allele.

The fact that *pal*G-21 leads to loss of PII and PII′ whilst *pal*G-23 leads to physical alteration of both of these enzymes and the nearly non-pleiotropic phenotype of *pal*G$^-$ mutations strongly imply that *pal*G is a structural gene for PII and PII′. Haploidisation analysis (McCully & Forbes, 1965) was used to locate *pal*G to linkage group III and meiotic analysis enabled its localization to a position on the left arm of this linkage group (Fig. 5).

(iii) pac*A is a structural gene for the phosphate-repressible acid phosphomonoesterase PV*

The principal phosphate-repressible acid phosphatase of *A. nidulans* PV was identified electrophoretically and partially purified and characterized by Harsanyi & Dorn (1972), but no mutations have been reported as affecting this enzyme. The electrophoresis patterns in Fig. 6 show that under phosphate-derepressing conditions, *pac*A-101 leads to loss of most of the acid phosphatase throughout the broad band of activity which corresponds to PV (lane 4 as compared to the wild type in lane 6). Under phosphate-repressing conditions there is relatively little difference (lane 10 as compared to lane 12). Data in Table 3 show that, after

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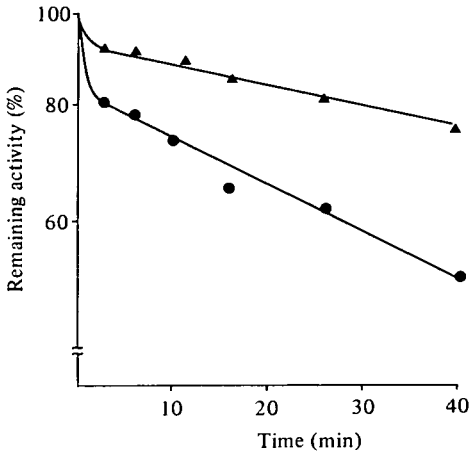

Fig. 2. Thermal inactivation of alkaline phosphatase PI in cell-free extracts at 60 °C. Mycelia were grown for 24 h at 25 °C in appropriately supplemented shaken liquid $-P_i$ medium to which (final concentrations) 200 μM phosphate and 20 mM ammonium were added. Alkaline phosphatase activity was assayed at pH 10. Each point is the mean of triplicate measurements from two independent experiments. ▲, *palD*$^+$ *palG*-21 strain; ●, *palD*-106 *palG*-21 strain.

growth at a non-permissive temperature, *pac*A-1 leads to a considerable reduction in phosphate-repressible acid phosphatase activity assayed at pH 6 in the presence of the cation chelator EDTA. If the assay pH is dropped to pH 4·6 and Mg^{2+} added rather than EDTA or if the assay pH is raised to pH 9·5, the effect of *pac*A-1 is much less pronounced. This is consistent with the reported cation-independence and pH optimum of 6·1 of PV (Harsanyi & Dorn, 1972).

Fig. 7 and 8 show that the thermosensitive alleles *pac*A-1 and *pac*A-100 lead to drastically reduced thermostability of the major acid phosphatase present under temperature-permissive, phosphate-derepressing growth conditions in cell extracts and media, respectively. Estimated half-lives are given in Table 4. As judged by colony staining, *pac*A$^-$ alleles are codominant with the wild type (*pac*A$^+$) allele.

The fact that *pac*A-101 (and at a non-permissive temperature *pac*A-1) lead to loss of PV whilst *pac*A-1 and *pac*A-100 lead to a physically altered enzyme and the lack of pleiotropy of *pac*A mutations strongly imply that *pac*A is a structural gene for PV. Dorn (1965*a*) located *pac*A to linkage group IV but was unsuccessful in attempting to locate it meiotically. Our further attempts to detect meiotic linkage have also been unsuccessful.

(iv) *Mutations in* pac*G lead to loss of non-repressible acid phosphomonoesterase*

No mutations resulting in loss of non-repressible acid phosphatase in *A. nidulans* have been reported. Unlike non-repressible alkaline phosphatase, non-repressible acid phosphatase in colonies can be stained without prior toluene-permeabilization. As judged by colony staining, *pac*G-92 and -110 and, at 42 °C, the thermosensitive mutation *pac*G-91 result in loss of acid phosphatase under conditions of phosphate sufficiency (i.e. on minimal medium). They have similar effects on acid phosphatase staining under conditions of phosphate limitation (i.e. in the presence of 50–200 μM phosphate) but no effect under conditions of phosphate starvation (i.e. on $-P_i$ medium). Under phosphate limitation they also result in lack of staining of the phosphate-repressible phosphodiesterase described by Brownlee *et al.* (1983) although they have no effect on phosphodiesterase activity in phosphate starvation conditions. (No phosphodiesterase activity is detectable in the wild type grown under phosphate sufficiency.) *pac*G$^-$ mutations do not affect staining for alkaline phosphatase under any growth conditions. The more extreme mutant alleles *pac*G-92 and -110 reduce rates of growth and conidiation at the optimal growth temperature 37 °C.

Electrophoretic patterns in Fig. 6 show that *pac*G$^-$ mutations lead to loss of a broad band of non-repressible acid phosphatase activity and that this phenotype is additive with that of a *pac*A$^-$ mutation. Despite the pronounced effects seen in Fig. 6, we have been unable to demonstrate more than a marginal effect of *pac*G$^-$ mutations (in *pac*A$^-$ or *pac*A$^+$ backgrounds) by enzyme assays. Possibly one or more of the activities

Table 2. *Codominance of* pal*D*$^-$ *and* pal*G*$^-$ *mutations*

Relevant genotype		Alkaline phosphatase
$-P_i$	*palG*-21 *palD*$^+$/*palG*-21 *palD*$^+$	61·0 ± 2·8
	palG-21 *palD*-8/*palG*-21 *palD*$^+$	38·0 ± 5·6
	palG-21 *palD*-8/*palG*-21 *palD*-8	1·0 ± 2·8
$+P_i$	*palG*$^+$ *palD*-8/*palG*$^+$ *palD*-8	8·7 ± 1·4
	palG-21 *palD*-8/*palG*$^+$ *palD*-8	5·2 ± 0·6
	palG-21 *palD*-8/*palG*-21 *palD*-8	0·0 ± 0

Cell-free extracts were prepared from mycelia grown for 12 h at 37 °C in appropriately supplemented shaken liquid minimal medium containing 20 mM (final concentration) ammonium as nitrogen source ($+P_i$) or additionally followed by 6 h in the same conditions but in phosphate-free medium ($-P_i$). Alkaline phosphatase activity was assayed at pH 10. Specific activities (± 1 S.D.) are expressed in nmoles *p*-nitrophenol liberated per mg soluble protein in extract per minute at 30 °C.

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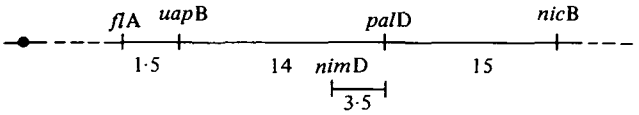

Fig. 3. Map position of *pal*D on right arm of linkage group VII. Map positions shown with distances in centimorgans are based on a number of crosses involving analysis of between 100 and 1000 progeny. Strains used carried various combinations of the mutant alleles *fl*A-1, *uap*B-70, *nim*D-4, *pal*D-8 and *nic*B-8. The relative order of *fl*A and *uap*B was confirmed by analysis of a cross of relevant partial genotype *uap*B-70 × *fl*A-1 *pal*D-8 *nic*B-8: of 18 *fl*A$^+$ *uap*B$^+$ recombinants, 13 carry *pal*D-8 and 12 carry *nic*B-8; of 6 *fl*A-1 *uap*B-70 recombinants, 1 carries *pal*D-8 and 2 carry *nic*B-8. The relative order of *nim*D and *pal*D was determined by analysis of a cross of relevant partial genotype *nim*D-4 × *fl*A-1 *pal*D-8 *nic*B-8: of 32 progeny selected as *nim*D$^+$ *pal*D$^+$, 19 are *fl*A-1 *nic*B$^+$, 8 are *fl*A$^+$ *nic*B$^+$, 3 are *fl*A-1 *nic*B-8 and 2 are *fl*A$^+$ *nic*B-8. Orientation with respect to the centromere (—●—) has been unequivocally established by mitotic and meiotic mapping experiments (R. I. Johnson, M. X. Caddick and H. N. Arst, Jr., unpublished results). Gene symbol definitions are given in Clutterbuck (1984). Efforts to detect meiotic linkage between the markers shown above and other linkage group VII markers have been unsuccessful.

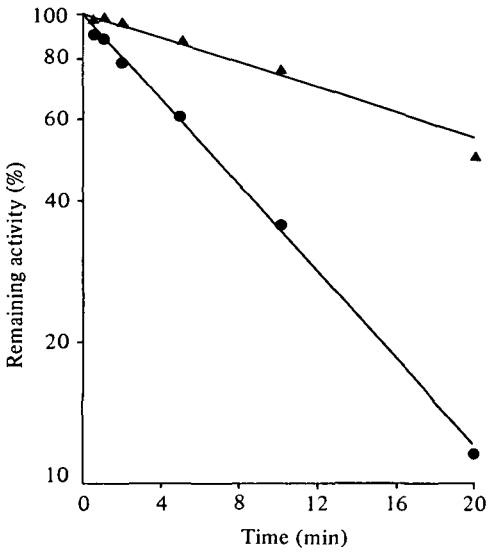

Fig. 4. Thermal inactivation of alkaline phosphatases PII and PII′ in cell-free extracts at 40 °C. Mycelia were grown for 24 h at 25 °C in appropriately supplemented shaken liquid minimal (high P_i) medium containing 20 mM (final concentration) ammonium as nitrogen source. Alkaline phosphatase activity was assayed at pH 9·5. Each point is the mean of triplicate measurements from two independent experiments. ▲, *pal*D-8 *pal*G$^+$ strain; ●, *pal*D-8 *pal*G-23 strain.

present fails to enter the gel or is inactivated by it. Inability to detect a pronounced effect of *pac*G$^-$ mutations in enzyme assays coupled with the considerable breadth of the electrophoretic band affected by these mutations would hinder the detection of physical alterations in non-repressible acid phosphatase resulting from leaky or conditional mutations.

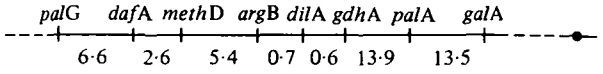

Fig. 5. Map position of *pal*G on left arm of linkage group III with respect to a number of loci centromere-proximal to it. Map positions shown with distances in centimorgans are based on a number of crosses involving analysis of between 340 and over 13000 progeny. Strains used carried various combinations of the mutant alleles *pal*G-21, *daf*A-7778, *meth*D-10, *arg*B-2, *dil*A-1, *gdh*A-10, *pal*A-1 and *gal*A-1. In addition to analysis of non-selected progeny upon which distances given above are largely based, the above gene order has been unequivocally confirmed by analysis of large numbers of selected rare recombinant classes of progeny. The position of the centromere (—●—) is from Clutterbuck (1984) where definitions of gene symbols other than *daf*A can be found. *daf*A (dicarboximide and aromatic hydrocarbon fungicide resistance) is the designation given by R. E. Beever (personal communication) to the locus where at least three of the mutations he selected (Beever, 1983) for iprodione resistance are located (H. N. Arst, Jr., unpublished results). The *daf*A mutations are allelic to the *chl*A mutations described by Tuyl (1977) and Martinez-Rossi & Azevedo (1982) and, as judged by their map position and cross-resistance to chlorinated nitrobenzenes and biphenyl, probably also to the (now lost) *pcnb*A mutations of Threlfall (1968; personal communication) (H. N. Arst, Jr., unpublished results). No attempt has been made to locate the markers shown above with respect to other linkage group III markers.

The effect of *pac*G$^-$ mutations on acid phosphatase and phosphodiesterase staining under phosphate-limitation bears some resemblance to an effect of certain purines, pyrimidines and nucleosides on wild-type strains. Growth under conditions of phosphate limitation (but not starvation or sufficiency) in the presence of adenosine, adenine, thymine or cytidine (but not hypoxanthine, guanine or uric acid) prevents staining for the phosphate-repressible enzymes, phosphodiesterase, acid phosphatase and, in contrast to the *pac*G$^-$ phenotype, alkaline phosphatase. Purine and pyrimidine derivatives have been implicated in phosphatase regulation in *Escherichia coli* (Wilkins, 1972) and *Neurospora crassa* (Hasunuma, 1977).

Colony staining experiments with strains carrying *pac*A$^-$ mutations show that the acid phosphatase produced under conditions of phosphate limitation in *pac*G$^+$ strains is the enzyme encoded by *pac*A. It is therefore likely that the primary effect of *pac*G$^-$ mutations is on the non-repressible acid phosphatase. The effect of *pac*G$^-$ mutations on phosphate-repressible acid phosphatase and phosphodiesterase might then result, for example, from participation of a substrate or product of a non-repressible acid phosphatase-catalysed reaction in the regulation of the phosphate-repressible enzymes.

Haploidization analysis located *pac*G to linkage group V. No attempt has been made to locate it further meiotically.

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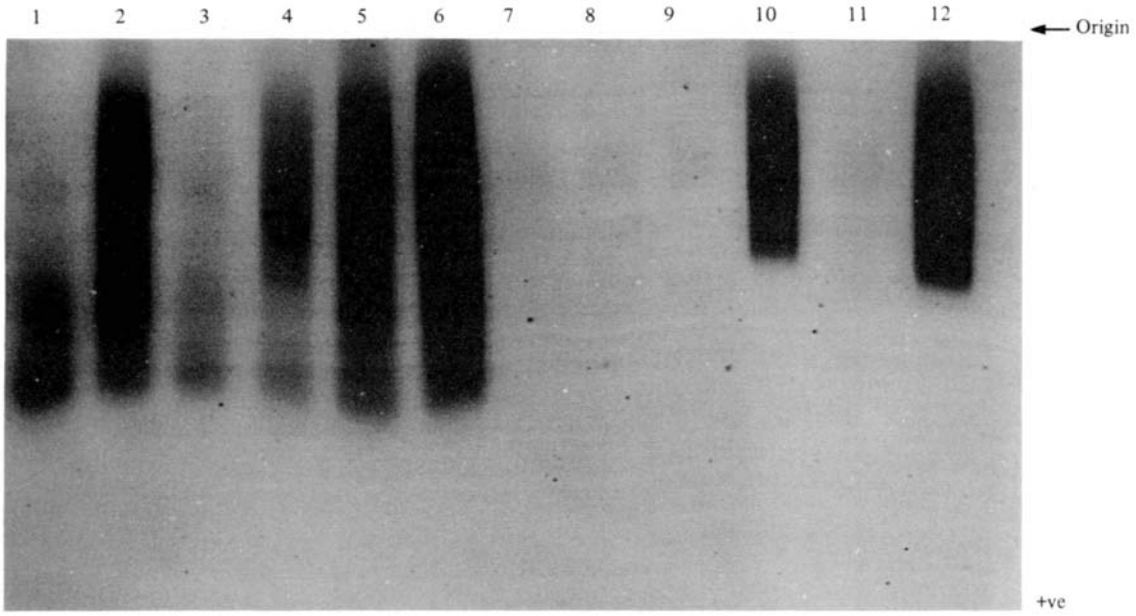

Fig. 6. Effects of *pac*A⁻ and *pac*G⁻ mutations on starch gel electrophoretic patterns of acid phosphatases. Mycelia were grown for 12 h at 37 °C in appropriately supplemented shaken liquid minimal medium containing 20 mM (final concentration) ammonium as nitrogen source (lanes 7–12) or additionally followed by 6 h in the same conditions but in phosphate-free medium (lanes 1–6). Lanes 1 and 7, *pac*A-101 *pac*G-110 strain; lanes 2 and 8, *pac*G-110 strain; lanes 3 and 9, *pac*A-101 *pac*G-92 strain; lanes 4 and 10, *pac*A-101 strain; lanes 5 and 11, *pac*G-92 strain; lanes 6 and 12, wild-type strain. The gel was stained for acid phosphatase activity only.

Table 3. *Effects of a* pac*A⁻ mutation on phosphatase activities*

	Phosphatase activity at					
	pH 6				pH 4·6	pH 9·5
	$+P_i$		$-P_i$		$-P_i$	
Relevant genotype	CE	M	CE	M	CE	CE
Wild type (*pac*A⁺)	7·1±0·9	< 0·1	338±26	30·7±4·1	118±11	135±2
*pac*A-1	6·8±0·2	< 0·1	43·4±3·6	1·9±0·3	49·8±4·2	105±8

Cell-free extracts (CE) and culture media (M) were assayed after growth of mycelia for 12 h at 37 °C in appropriately supplemented shaken liquid minimal medium containing 20 mM (final concentration) ammonium as nitrogen source ($+P_i$) or additionally followed by 6 h in the same conditions but in phosphate-free medium ($-P_i$). Alkaline phosphatase activity was assayed in 100 mM diethanolamine buffer at pH 9·5. Acid phosphatase activity was assayed in 100 mM maleate (as the Na^+ salt) buffer at pH 6 in the presence of 2 mM-EDTA or in 100 mM acetate (as the Na^+ salt) buffer at pH 4·6 in the presence of 10 mM-$MgCl_2$. Specific activities (±1 S.D.) are expressed in nmoles *p*-nitrophenol liberated per mg soluble protein in extract per minute (CE) or nmoles *p*-nitrophenol liberated per mg dry weight per minute (M), both at 30 °C. Soluble protein in extracts accounts for approximately 12% of mycelial dry weight (Brownlee & Arst, 1983).

(v) pac*C is not a structural gene for a phosphatase*

An earlier report from this laboratory (Arst, Bailey & Penfold, 1980) suggested that *pac*C might be a structural gene for the phosphate-repressible acid phosphatase designated PIV by Dorn (1965*a*, *b*). This suggestion was based on a photograph (Dorn, 1965*b*) of an electrophoresis gel in which the thermosensitive allele *pac*C-5 apparently led to an alteration in electrophoretic mobility of PIV after growth at a permissive temperature. We have been unable to repeat this observation, either using *pac*C-5 or any of several other leaky or conditional *pac*C mutant alleles. In every gel where PIV from a mutant strain is observable, its mobility does not differ significantly from that of the wild type enzyme. The highly pleiotropic phenotype of *pac*C mutations has been reported previously (Arst & Cove, 1970; Arst *et al.* 1980). Results to be presented elsewhere (M. X. Caddick, A. G. Brownlee & H. N. Arst, Jr., in preparation) will show that *pac*C mutations affect PV and phosphate-repressible phosphodiesterase in a fashion similar to PIV and that *pac*C is a regulatory gene involved in the control of several permeases as well as phosphatases.

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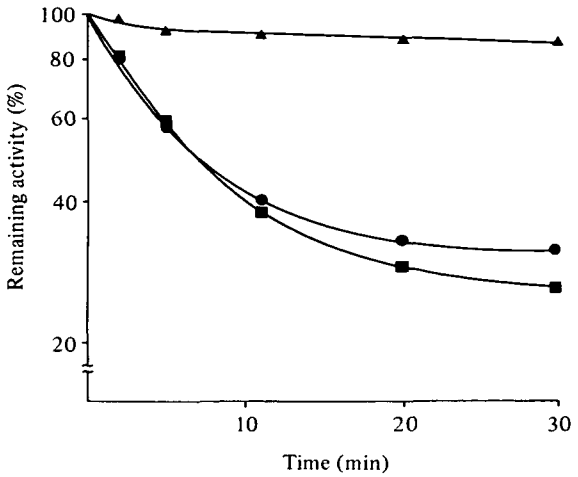

Fig. 7. Thermal inactivation of acid phosphatase PV in cell-free extracts at 40 °C. Mycelia were grown for 24 h at 25 °C in appropriately supplemented shaken liquid $-P_i$ medium to which (final concentrations) 200 μM phosphate and 20 mM ammonium were added. Acid phosphatase was assayed in 100 mM maleate (as the Na^+ salt) buffer at pH 6 in the presence of 2 mM-EDTA. Each point in the mean of triplicate measurements from two independent experiments. ▲, wild type (*pac*A^+) strain; ■, *pac*A-100 strain; ●, *pac*A-1 strain.

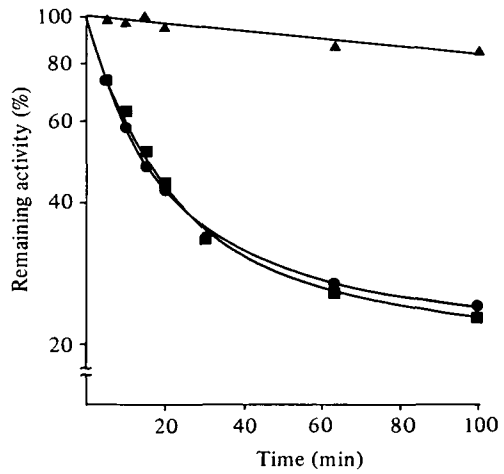

Fig. 8. Thermal inactivation of acid phosphatase PV in culture medium at 50 °C. Culture media were collected after mycelia were grown for 24 h at 25 °C in appropriately supplemented shaken liquid $-P_i$ medium to which (final concentrations) 200 μM phosphate and 20 mM ammonium were added. Acid phosphatase was assayed in 100 mM maleate (as the Na^+ salt) buffer at pH 6 in the presence of 2 mM-EDTA. Each point is the mean of triplicate measurements from two independent experiments. ▲, wild type (*pac*A^+) strain; ■, *pac*A-100 strain; ●, *pac*A-1 strain.

(vi) *The phosphatases encoded by* pal*D*, pal*G*, pac*A and putatively by* pac*G are dispensable*

Doubly, triply and quadruply mutant strains have been constructed with *pal*D^-, *pal*G^-, *pac*A^- and *pac*G^- alleles in all combinations. In each case the phenotypes are those predicted from additivity of the phenotypes of individual mutations. Thus none of these phosphatases plays an essential role, even in the absence of others.

Table 4. *Effects of thermosensitive* pacA^- *alleles on half-lives of acid phosphatase PV*

Relevant genotypes	$t_{\frac{1}{2}}$ (min) CE	M
Wild type (*pac*A^+)	62	350
*pac*A-1	3·5	8·5
*pac*A-100	4·0	9·5

The extrapolated relatively thermostable residual activities (i.e. the activities on the nearly horizontal asymptotes) were substracted from the curves shown in Fig. 7 (CE) and 8 (M) to yield straight-line semi-logarithmic plots from which the above half-lives ($t_{\frac{1}{2}}$) were estimated.

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