Mol Gen Genet (1986) 203:346–353

MGG

Regulation of gene expression by pH of the growth medium in *Aspergillus nidulans*

Mark X. Caddick*, Alan G. Brownlee, and Herbert N. Arst, Jr.***
Department of Genetics, Ridley Building, The University, Newcastle upon Tyne NE1 7RU, England

Summary. In the fungus *Aspergillus nidulans* the levels of a number of enzymes whose location is at least in part extracellular (e.g. acid phosphatase, alkaline phosphatase, phosphodiesterase) and of certain permeases (e.g. that for γ-amino-*n*-butyrate) are controlled by the pH of the growth medium. For example, at acidic pH, levels of acid phosphatase are high and those of alkaline phosphatase are low whereas at alkaline pH the reverse is true. Mutations in five genes, *palA*, *B*, *C*, *E* and *F*, mimic the effects of growth at acid pH whereas mutations in *pacC* mimic the effects of growth at alkaline pH. *palA*, *B*, *C*, *E* and *F* mutations result in an intracellular pH (pH_{in}) which is more alkaline than that of the wild type whereas *pacC* mutations result in a pH_{in} more acidic than that of the wild type. This indicates that these mutations exert their primary effects on the regulation of gene expression by pH rather than on the pH homeostatic mechanism but that the expression of at least some component(s) of the pH homeostatic mechanism is subject to the pH regulatory system. It is suggested that *pacC* might be a wide domain regulatory gene whose product acts positively in some cases (e.g. acid phosphatase) and negatively in others (e.g. alkaline phosphatase). The products of *palA*, *B*, *C*, *E* and *F* are proposed to be involved in a metabolic pathway leading to synthesis of an effector molecule able to prevent the (positive and negative) action of the *pacC* product.

These genes are, to our knowledge, the first examples of genes involved in the regulation of extracellular enzyme and permease synthesis by the pH of the growth medium to be described in any organism.

Key words: *Aspergillus nidulans* – Extracellular enzymes – Gene regulation – Permeases – pH regulation

Introduction

The ascomycete fungus *Aspergillus nidulans* is a nutritionally versatile organism able to grow over a wide range of growth conditions, including pH. Wild type strains of *A. nidulans* are able to grow in media as acidic as pH 3.5 or as basic as pH 9. Nevertheless, as shown here, this organism has an efficient pH homeostatic mechanism so that most enzymes are required to act only over a rather narrow pH range. Extracellular enzymes and permeases, however, are not thus protected from relative extremes of pH. It is not therefore difficult to see the utility of a regulatory system which ensures that extracellular enzymes and permeases are synthesised only at pH values where they can function effectively. For example, as shown here, *A. nidulans* is able to synthesise acid phosphatase predominantly at acid pH and alkaline phosphatase predominantly at alkaline pH. The existence of a mechanism for regulation of extracellular enzyme synthesis by the pH of the growth medium has been noted for several micro-organisms including *A. nidulans* (Cohen 1980; Lindberg et al. 1982; Nahas et al. 1982; Gander and Janovec 1984; Kobayashi et al. 1984; Caddick et al. 1986). Here the range and extent of such pH regulation in *A. nidulans* are further characterised. More importantly, for the first time in any organism, mutations are described which alter the regulation of the synthesis of extracellular enzymes and permeases by growth pH. These fall into two categories, those which mimic the effects of growth at acidic pH and those which mimic the effects of growth at alkaline pH.

Materials and methods

Strains and growth testing. A. nidulans strains used in this work carried markers which have been described previously (Dorn 1965a, b; Arst and Cove 1970; Arst et al. 1980; Clutterbuck 1984; Caddick and Arst 1986; Caddick et al. 1986) with the exceptions of *palB-111* and *pacC-14*. *palB-111* was selected after N-methyl-N′-nitro-N-nitrosoguanidine mutagenesis (Alderson and Hartley 1969) of a strain of genotype *biA-1 areA′-2 fw*A-1 (biotin-requiring, unable to utilise nitrogen sources other than ammonium, having fawn conidial colour) as suppressing *areA′-2* for utilisation of 5 mM γ-amino-*n*-butyrate as sole nitrogen source (see Results section) at pH 6.5. *palB-111* is recessive to $palB^+$ in heterozygous diploids. It is tightly linked (<0.01 cM) to *palB-7* and fails to complement *palB-7* in a heteroallelic diploid. *pacC-14* was selected after ultraviolet mutagenesis of a strain of genotype *pabaA-1 gatA-2 palF-15 fw*A-1 (*p*-aminobenzoate-requiring, lacking ω-amino acid transaminase, mimicking growth at acidic pH (vide infra), having

* *Present address:* Department of Bacteriology, Royal Postgraduate Medical School, Hammersmith Hospital, Ducane Road, London W12 OHS, England
** *Present address:* CSIRO Division of Animal Production, P.O. Box 239, Blacktown, NSW 2148, Australia
Offprint requests to: H.N. Arst

fawn conidial colour) as relieving the toxicity of 5 mM γ-amino-*n*-butyrate due to the *gatA-2* mutation (Arst 1976; Arst et al. 1978; Arst et al. 1980) in the presence of 10 mM ammonium (as the (+)-tartrate) as nitrogen source. All strains used for enzyme or uptake assays (including electrophoretic analysis) or intracellular pH determinations carry the *p*-aminobenzoate auxotrophy *pabaA-1*. The *palB-7* strain used for γ-amino-*n*-butyrate uptake studies and intracellular pH determinations (but not for enzyme assays) also carries *chaA-1* (chartreuse conidial colour). Growth testing has been described by Arst and Cove (1969) and Arst et al. (1982). The solid minimal medium of Cove (1966) was used. This medium is phosphate-sufficient and its pH is 6.5. The pH 5 and phosphate-buffered pH 8 solid media of Cove (1976) were used. Both of these media are phosphate-sufficient. Phosphate-free (-P_i) medium was prepared by substituting chloride salts for phosphate salts at equimolar cation concentrations. It was buffered at pH 5 or 6.5 with 20 mmol l^{-1} citrate (as the sodium salts) or at pH 8 with 20 mmol l^{-1} tris-HCl. Unless otherwise indicated 1% (w/v) D-glucose was present as carbon source and 10 mM ammonium (as the (+)-tartrate) was used as nitrogen source. Inoculated solid media were incubated at 37° C.

Colony staining, starch gel electrophoresis and enzyme assays. Detection of acid and alkaline phosphatases and phosphodiesterases by colony staining has been described previously as have starch gel electrophoresis and assay of these enzymes (Dorn 1965a; Caddick and Arst 1986; Caddick et al. 1986). Acid phosphatase assays were carried out at pH 6 in the presence of 2 mM EDTA. These assay conditions monitor mainly acid phosphatase PV (Harsanyi and Dorn 1972; Caddick and Arst 1986). Alkaline phosphatase was assayed at pH 10 in the presence of 10 mM $MgCl_2$. Phosphodiesterase was determined using *p*-nitrophenyl phenylphosphonate as substrate. 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 min at 30° C. For comparison of intra- and extracellular enzyme levels, note that soluble protein in extracts accounts for approximately 12% of mycelial dry weight (Brownlee and Arst 1983).

The liquid minimal medium of Cove (1966) was used. It is phosphate-sufficient with a pH of 6.5. -P_i liquid medium was prepared by substituting chloride salts for phosphate salts at equimolar cation concentrations. Unless otherwise indicated, 1% (w/v) D-glucose was present as carbon source and 20 mM ammonium (as the (+)-tartrate) was used as nitrogen source.

γ-amino-n-butyrate (GABA) uptake measurements. GABA uptake assays were based on methods described by Bailey et al. (1979) and Brownlee and Arst (1984). To avoid effects of pH on induction efficiency, exogenous co-inducers were not present. However, all strains used for uptake studies carry *gatA-2*, resulting in loss of GABA transaminase and preventing GABA metabolism (Arst 1976; Bailey et al. 1980). *gatA-2* leads to partial induction of GABA permease due to accumulation of endogenous co-inducer(s) (Bailey et al. 1980). Mycelia were grown for 7 h at 37° C in appropriately supplemented shaken liquid minimal medium (Cove 1966) containing (final concentrations) 1% (w/v) D-glucose as carbon source and 100 mg/l uric acid as nitrogen source buffered at pH 5 or 6.5 with 50 mM citrate (Na^+) or at pH 8 with 50 mM tris-HCl. Mycelia were then harvested on Miracloth (Calbiochem, La Jolla, CA, USA), washed with 200 ml and resuspended in 20 ml of ice-cold unbuffered but otherwise identical medium having the same pH. Kept ice-cold and with gentle stirring, no change in GABA uptake activity occurred for at least 2 h. One millilitre aliquots of mycelial suspensions were then added to 9 ml pre-warmed (37° C) appropriately supplemented liquid minimal medium containing (final concentrations) 1% (w/v) D-glucose as carbon source and 100 mg/l uric acid as nitrogen source buffered at pH 5 or 6.5 with 50 mM citrate (Na^+) or at pH 8 with 50 mM tris-HCl and shaken for 12 min at 37° C to allow temperature equilibration. [U-^{14}C] GABA (Amersham International, Amersham, Bucks., UK) was then added to a final concentration of 200 μM at a final specific activity of 50 mCi mol^{-1}. After 4 min mycelia were harvested on 2.5 cm diameter Whatman GF/A glass fibre discs and washed with 10 ml 100 mM unlabelled GABA under vacuum. Assays in the presence of (final concentration) 1 mM KCN were used to correct for the presence of label not due to active uptake. Using this method GABA uptake was linear for at least 10 min. Discs with mycelia were then placed into scintillation vials to which 800 μl H_2O and 10 ml scintillant [1.5 l toluene, 500 ml Triton X-100, 8 g 2-(4-*tert*-butylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole] were added. The mixture was vortexed twice for 30 s and allowed to settle before counting in a Beckman LS-333 liquid scintillation counter. Counting and quench correction followed the procedure of Brownlee and Arst (1984), using acetone as quenching agent. Mycelial dry weights were determined from two 1 ml aliquots of mycelial suspensions, taken at the beginning and end of the uptake experiment, harvested on pre-weighed glass fibre discs. These were dried overnight at 80° C and re-weighed.

Intracellular pH (pH_{in}) determinations. Mycelia were grown for 16 h at 37° C in appropriately supplemented shaken liquid minimal medium containing (final concentrations) 1% (w/v D-glucose as carbon source and 20 mM ammonium (as the (+)-tartrate) as nitrogen source, buffered at pH 6.5 with 50 mM citrate (Na^+). Mycelia were washed with 1% (w/v) D-glucose at 37° C, filtered and suspended in 50 ml buffer [200 mM citrate (Na^+) at pH 3.5 or 5.0, 200 mM 2-(N-morpholino)ethanesulphonic acid (Na^+) at pH 6.5 or 200 mM tris-HCl at pH 8.0] containing (final concentration) 1% (w/v) D-glucose and shaken at 37° C for 30 min. Following incubation mycelia were filtered and washed with distilled water. One to three gramme wet weight samples were transferred to 20 ml 0.1% (v/v) Triton X-100 in duplicate, frozen at −80° C overnight and disrupted by rapid thawing to room temperature. The pH values were then determined immediately using a Philips PW 9420 pH meter. Duplicate determinations agreed within 0.1 pH unit. Three completely independent experiments with the wild type strain yielded essentially identical results.

Results

The extent and range of regulation by growth pH

It is not difficult to observe examples of the regulation of extracellular enzyme or permease synthesis by the pH of the growth medium in *A. nidulans* colonies growing on

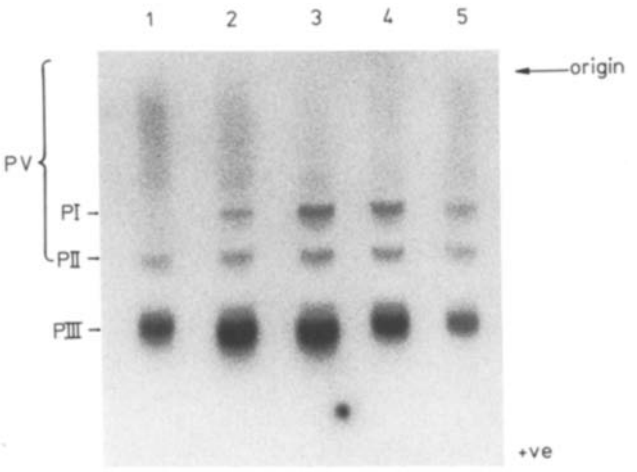

Fig. 1. The effect of growth pH on starch gel electrophoresis patterns of acid and alkaline phosphatases in a wild type strain. Mycelia were grown for 15 h at 37° C in appropriately supplemented shaken -P_i liquid medium to which 200 μmol/l phosphate was added. The medium was buffered at pH 4.5 (lane 1), 5.5 (lane 2) or 6.5 (lane 3) with 50 mM citrate (Na^+) or at pH 7.5 (lane 4) or 8.5 (lane 5) with 50 mM tris-HCl. Whatman no. 3 filter papers were saturated with cell free extracts and loaded into gel slots. Samples were not standardised for protein concentration in order to minimise activity loss during manipulations. 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. For further details of starch gel electrophoresis of the phosphatases shown see Dorn (1965a, b), Harsanyi and Dorn (1972) and Caddick and Arst (1986)

solid media. On phosphate-free (-P_i) media, wild type colonies stain most intensely for alkaline phosphatase when growing at pH 8, less intensely when growing at pH 6.5 and not at all when growing at pH 5.[1] The reverse is true when acid phosphatase or phosphodiesterase (which in this organism has an acidic pH optimum) is stained, with weakest staining in colonies growing at pH 8 and strongest in colonies growing at pH 5. Colony staining in the absence of permeabilisation (as in these cases) indicates that the activity is at least partially extracellular (Caddick and Arst 1986). Utilisation of γ-amino-*n*-butyrate (GABA) as a carbon (usually at 50 mM) and/or nitrogen (usually at 5 mM) source provides another example, being strongest at pH 5 and virtually non-existent at pH 8. A means of examining the basis for this pH effect on GABA utilisation is provided by *areA*^{r} mutants. *areA* is a positive-acting wide domain regulatory gene mediating nitrogen metabolite repression and its product is required for the syntheses of many enzymes and permeases involved in nitrogen source utilisation (Arst and Cove 1973; Wiame et al. 1985). Loss-of-function mutations, designated *areA*^{r}, lead to inability to utilise nitrogen sources other than ammonium. In the case of GABA utilisation, it has been shown that lack of GABA uptake is responsible (Bailey et al. 1979). *areA*^{r} mutants are unable to utilise GABA as a nitrogen source at the standard growth pH of 6.5 (or at pH 8) but can do so at pH 5. Toxicities of neomycin (at 1–3 mg/ml) and kanamycin (at 3–5 mg/ml) are also strongly influenced by pH, increasing as the pH is raised from 5 to 8. Molybdate toxicity (tested at 33 mM) follows the reverse pH dependence, being less toxic at pH 8 than at pH 6.5. Below pH 6 molybdate condenses to form polymolybdates (Cotton and Wilkinson 1962), precluding toxicity testing at low pH.

Evidence for pH regulation can also be seen using starch gel electrophoresis and enzyme and uptake assays. Figure 1 shows that the level of the phosphate-repressible alkaline phosphatase PI (Dorn 1965a, b; Caddick and Arst 1986; Caddick et al. 1986) increases as the growth pH increases from 4.5 to 8.5 whereas the reverse is true of the phosphate-repressible acid phosphatase PV (Harsanyi and Dorn 1972; Caddick and Arst 1986; Caddick et al. 1986). No pH effect is seen for certain other phosphatases including the intracellular and possibly inducible alkaline phosphatase PII (Caddick and Arst 1986; Caddick et al. 1986). Data in Table 1 quantitatively confirm these conclusions for PI and PII. In a wild type strain growing in nutrient-sufficient or nitrogen starvation conditions where PII (and the related enzyme PII′) is the predominant alkaline phosphatase no effect of growth pH is apparent, whereas in phosphorus starvation conditions where PI accounts for about half the alkaline phosphatase activity, pH regulation is evident. pH regulation is much more pronounced in a *palG-21* strain. *palG-21* is a structural gene mutation leading to loss of PII and PII′ (Caddick and Arst 1986). In contrast, in a *palD-8* strain no pH regulation is seen. *palD-8* is a structural gene mutation leading to loss of PI (Caddick and Arst 1986). Figure 2 shows the pH dependence of levels of intra- and extracellular activities of alkaline phosphatase (Fig. 2A and B), acid phosphatase (Fig. 2C and D) and phosphodiesterase (Fig. 2E and F) under phosphate-derepressing conditions. The principal phosphate-repressible acid phosphatase assayed at pH 6 in the presence of EDTA is PV (Harsanyi and Dorn 1972; Caddick and Arst 1986). The wild type shows very marked pH regulation of acid phosphatase levels as well as those of alkaline phosphatase and phosphodiesterase.

Data in Fig. 3 confirm pH regulation of GABA uptake levels. The principal GABA permease is specified by the *gabA* gene (Bailey et al. 1979). If the *gabA*-dependent GABA uptake activity is calculated (by subtracting uptake activity in a *gabA*$^-$ strain from that in an otherwise isogenic *gabA*$^+$ strain), the pH regulation is much more pronounced (Fig. 3). The level of *gabA* expression is greatest at pH 5 and lowest at pH 8, correlating well with the effect of pH on permease activity, highest assayed at pH 5 and lowest assayed at pH 8 (Fig. 3).

In growth media near pH 6.5, mutations in palA, palB, palC, palE and palF can mimic the effects of growth at more acidic pH

Mutations in *palA*, *B*, *C*, *E* and *F* result in loss of alkaline phosphatase PI, judged by starch gel electrophoresis, and a lack of colony staining for alkaline phosphatase on -P_i medium at pH 6.5 (Dorn 1965a, b). In contrast they enhance colony staining for acid phosphatase (Dorn 1965a) and phosphodiesterase on the same growth medium. Enhanced acid phosphatase staining appears to result mostly from increased levels of PV because the PV structural gene loss-of-function mutation *pacA-101* eliminates it, *palB-7*

1 In interpreting these results, it is important to recall that the staining mixtures (Dorn 1965a; Caddick and Arst 1986) have a much stronger buffering capacity than the growth media

Table 1. Alkaline phosphatase levels as a function of growth pH in nutrient starvation and sufficiency

Relevant genotype	Specific activities in media of nutrient status and pH								
	MM			-N			-P		
	5	6.5	8	5	6.5	8	5	6.5	8
Wild type	12.1±0.8	14.2±0.6	12.6±0.2	65.7±6.1	61.8± 0.0	62.6± 4.4	54.0±2.1	85.6±6.2	125±1
palG-21	n.d.	0.8±0.1	n.d.	n.d.	0.1± 0.2	n.d.	1.7±2.3	47.9±8.2	77.1±5.1
palD-8	n.d.	13.9±0.4	n.d.	61.9±5.4	53.8±11.2	49.6±10.0	53.0±1.4	45.3±9.1	51.4±3.7
palG-21 palD-8	n.d.	0.1±0.1	n.d.	n.d.	<0.1	n.d.	0.1	<0.1	0.1

Alkaline phosphatase was assayed in cell-free extracts after growth of mycelia for 12 h at 37° C in appropriately supplemented shaken liquid minimal medium (MM) or after transfer in otherwise identical conditions to medium lacking a nitrogen source (-N) for 4 h or to -P_i medium (-P) for 6 h. Media were buffered at pH 5 or 6.5 with 50 mM citrate (Na^+) or at pH 8 with 50 mM tris-HCl. Mycelia which were transferred to -N or -P conditions were initially grown in MM buffered at pH 6.5. Specific activities ± one standard deviation are given except where <0.1. Where specific activities are given as 0.1, these are single determinations. n.d. = not determined

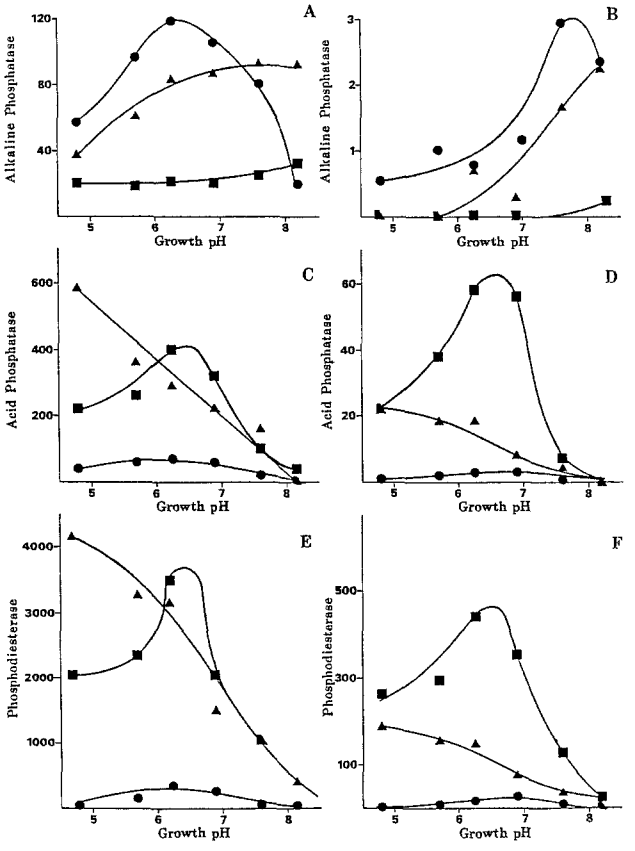

Fig. 2A–F. The variations in alkaline phosphatase (**A** and **B**), acid phosphatase (**C** and **D**) and phosphodiesterase (**E** and **F**) levels in cellfree extracts (**A**, **C** and **E**) and culture media (**B**, **D** and **F**) in a wild type (▲), a *pacC-5* (●) and a *palB-7* (■) strain as a function of growth pH. Mycelia were grown for 12 h at 37° C in appropriately supplemented, shaken liquid minimal medium and then transferred to buffered -P_i liquid medium for 4 h in otherwise identical conditions. -P_i media were buffered at pH 4.8 or 5.7 with 50 mM citrate (Na^+), at pH 6.3 with 50 mM 2-(N-morpholino)ethanesulphonic acid (Na^+), at pH 7.0 with 50 mM piperazine-N,N′-bis(2-ethanesulphonic acid) (Na^+) or at pH 7.7 or 8.3 with 50 mM tris-HCl. The growth pHs plotted are the averages of initial and final values

pacA-101 double mutants being indistinguishable from *pacA-101* single mutants for acid phosphatase staining. As expected from starch gel electrophoresis patterns (Dorn 1965a, b), the PI structural gene mutation *palD-8* is only detectable in *palB-7 palD-8* double mutants upon outcrossing whereas the PII (and PII′) structural gene mutation *palG-21* is additive in double mutants with *palA-1* and *palB-7*. (Other double mutant combinations have not been constructed.) In strains carrying a functional *gabA* allele, *palA*, *B*, *C*, *E* and *F* mutations suppress *areA*^{r} mutations for utilisation of 5 mM GABA as nitrogen source at pH 6.5. In *gabA*$^+$ *areA*$^+$ strains they enhance utilisation of GABA as carbon and/or nitrogen source. At pH 6.5 they result in hypersensitivity to molybdate toxicity (Arst and Cove 1970) but confer some resistance to neomycin and kanamycin toxicities.

The above in vivo observations are in strong agreement with data in Table 2 and Figs. 2 and 3. When grown in phosphate-derepressing conditions at pH 6.3, a *palB-7* strain has reduced intra- and extracellular levels of alkaline phosphatase but enhanced levels of acid phosphatase and phosphodiesterase (Table 2). In phosphate-sufficient growth conditions *palB-7* has no effect on phosphatase levels (data not shown). When growth pH profiles over the range 4.8 to 8.2 in phosphate-derepressing conditions are compared (Fig. 2), the patterns are more complex but nevertheless confirm that in the pH 6 to 7 range *palB-7* reduces both intra- and extracellular levels of alkaline phosphatase whilst increasing those of acid phosphatase and phosphodiesterase. Data in Fig. 3 show that in growth media buffered at pH 6.5 or 8 *palB-7* leads to considerably enhanced levels of the *gabA* permease.

All of these observations are consistent with the formal hypothesis that *palA*, *B*, *C*, *E* and *F* mutations in a growth medium near pH 6.5 mimic the effect of growth around pH 5.

In growth media near pH 6.5, mutations in pacC can mimic the effects of growth at more alkaline pH

Mutations in *pacC* can result in reduced colony staining for acid phosphatase (Dorn 1965a, b) and phosphodiester-

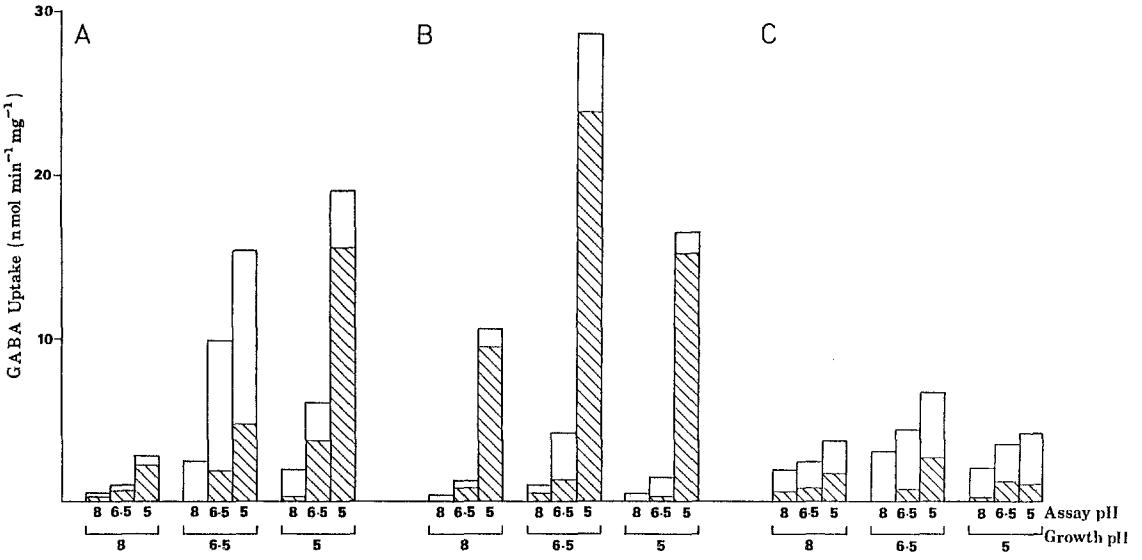

Fig. 3A–C. GABA uptake activity as a function of growth pH and assay pH in wild type (**A**), *palB-7* (**B**) and *pacC-5* (**C**) strains. The total height (*hatched plus non-hatched*) of each bar is the uptake activity of a *gabA*$^+$ strain of each genotype. The *non-hatched* distance in each bar is the activity of a *gabA-2* strain of each genotype. *gabA-2* is a loss-of-function mutation (Bailey et al. 1979) and therefore the remaining, *hatched* height represents the activity due to the *gabA*-specified permease. The data shown are the average of at least three independent experiments

Table 2. The effects of a *palB* mutation and a *pacC* mutation on phosphatase levels in phosphate-derepressing growth conditions

Relevant genotype	Alkaline phosphatase		Acid phosphatase		Phosphodiesterase	
	CE	M	CE	M	CE	M
Wild type	81.8±8.2	0.7±0.2	292 ±31	17.8±0.4	3150±470	150 ±20
palB-7	21.1±1.2	<0.1	402 ±11	58.5±7.5	3530±250	441 ±50
pacC-5	119 ±5	0.8±3	71.2± 2.9	2.1±0.3	327±1	14.9±4

Mycelia were grown for 12 h at 37° C in appropriately supplemented shaken liquid minimal medium and transferred to -P_i medium buffered at pH 6.3 with 50 mM 2-(N-morpholino)ethanesulphonic acid (Na^+) for a further 4 h under otherwise identical growth conditions. Specific activities ± one standard deviation are given except where <0.1

ase but enhanced staining for alkaline phosphatase on -P_i medium at pH 6.5. They can reduce GABA uptake and partially prevent GABA utilisation (Arst et al. 1980). They can result in resistance to molybdate toxicity (Arst and Cove 1970) and in hypersensitivity to neomycin and kanamycin toxicities.

In phosphate-derepressing growth conditions at pH 6.3, *pacC-5* leads to reduced intra- and extracellular levels of acid phosphatase and phosphodiesterase and to a slightly elevated intracellular alkaline phosphatase level (Table 2). In phosphate-sufficient growth conditions *pacC-5* has no effect on phosphatase levels (data not shown). Over a wide range of growth media pH, *pacC-5* considerably reduces levels of phosphate-repressible acid phosphatase and phosphodiesterase activities but in the acidic to neutral pH range it elevates phosphate-repressible alkaline phosphatase levels (Fig. 2). Irrespective of growth pH, *pacC-5* leads to very low levels of the *gabA* permease (Fig. 3).

The above observations accord with the formal hypothesis that in growth media near pH 6.5, *pacC* mutations have a phenotype similar to the effects of growth at around pH 8 on a wild type strain.

One further aspect of the *pacC* mutant phenotype might indicate an additional activity affected by *pacC* mutations. Double mutants carrying putative loss-of-function mutations in *pacJ* and *palcA* are unable to grow on solid minimal (phosphate-sufficient) medium at pH 5 and do so only poorly at pH 6.5, probably because of defective phosphate uptake (Caddick et al. 1986). *pacC* mutations overcome this defect as judged by growth of *pacC-5 pacJ-121 palcA-1* triple mutants under these conditions. Once again an effect of growth at pH 8 (where *pacJ palcA* double mutants can grow normally on minimal medium) is mimicked by *pacC* mutations. *pacC* mutations would therefore appear also to affect levels of a phosphate permease.

Mutations in palB (and presumably also palA, B, C, E and F) and pacC affect pH_{in}

One possible reason why mutations might mimic the effects of growth at another pH would be if they alter intracellular pH (pH_{in}), assuming that pH_{in} varies as a function of growth medium pH (confirmed below) and further that the regulatory system mediating response to growth pH actually monitors pH_{in} rather than external pH. Data in Fig. 4 show how bulk pH_{in} varies as a function of extracellular pH for wild type, *pacC-5* and *palB-7* strains over the range 3.5 to 8. *pacC-5* results in a consistently more acidic pH_{in}

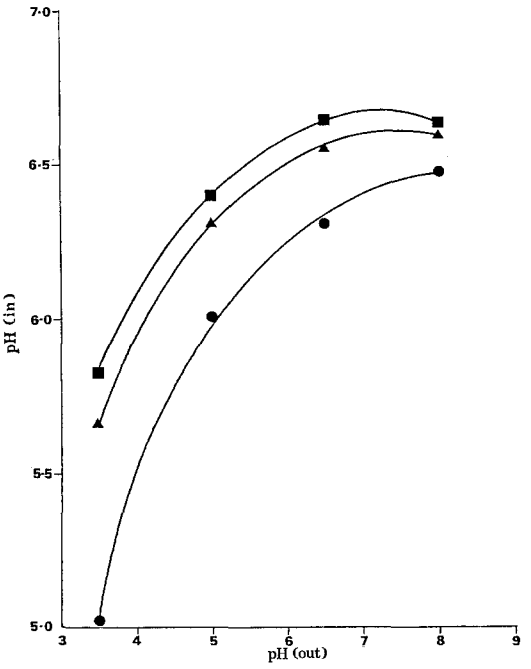

Fig. 4. Intracellular pH (pH_{in}) as a function of external pH (pH_{out}) in a wild type (▲), a *pacC-5* (●) and a *palB-7* (■) strain

and *palB-7* in a consistently more alkaline pH_{in} over the entire range. Less complete data (not shown) indicate that the more extreme mutant allele *pacC-14* leads to an even more acidic pH_{in} than *pacC-5*. The parallel responses of these strains to changes in external pH indicate that *palB* and *pacC* mutations do not abolish pH homeostasis or at least that they allow maintenance of a pH gradient across the plasma membrane.

These effects on pH_{in} might explain some growth characteristics. Non-leaky *palA*, *B*, *C*, *E* and *F* mutations result in inability to grow on alkaline (e.g. pH 8) solid media. Perhaps this is a consequence of a pH_{in} too alkaline to permit growth. Whatever the reason, it provides a convenient method for scoring these mutations and an excellent method for selecting revertants. *pacC* mutations result in abnormal morphology with reduced conidiation at acidic pH. This might be a consequence of an abnormally low pH_{in} in acidic media.

One further point should be noted from Fig. 4. At or just above pH 6.5, pH_{in} equals external pH in a wild type strain. Although the optimal pH for growth does vary according to the nutrients supplied (section 1 of Results; Cove 1976), pH 6.5 is usually optimal for growth (unpublished observations) and is the standard pH for *A. nidulans* media (Cove 1966). This is probably pertinent to the fact that it is for growth in media near pH 6.5 when *pacC* mutations mimic alkaline growth conditions most completely and *palA*, *B*, *C*, *E* and *F* mutations mimic acidic growth conditions most completely.

Mutations in pacC can suppress palA, B, C, E and F mutations for all aspects of their phenotype

Double mutants carrying *palA-1*, *palB-7*, *palB-111*, *palC-4*, *palE-11* or *palF-15* in addition to *pacC-5* are phenotypically indistinguishable from *pacC-5* single mutants, the *pal* mutations being identifiable only upon outcrossing. Triple mutants carrying *palA-1*, *palB-7*, *palB-111*, *palC-4*, *palE-11* or *palF-15* in addition to *pacC-5* and an *areaA*^{r} mutation are similarly indistinguishable from *pacC-5 areaA*^{r} double mutants. Furthermore the mutation *suB-2palB-7* selected by Dorn (1965a) is a *pacC* allele as judged by lack of complementation with *pacC-5* in a diploid and tight linkage (< 0.01 cM).

Although *pacC* mutations restore alkaline phosphatase PI to *palB-7* strains as judged by starch gel electrophoresis (Dorn 1965a) and to all *palA*, *B*, *C*, *E* or *F* mutant strains as judged by colony staining, they do not suppress loss-of-function mutations in *palD*, the structural gene for PI (Caddick and Arst 1986), or in *palcA*, the positive-acting wide domain regulatory gene mediating phosphate repression (Caddick et al. 1986). Moreover, *pacA-1*, a loss-of-function mutation in the acid phosphatase PV structural gene (Caddick and Arst 1986), does not suppress *palC-4* or *palF-15* (and presumably therefore any other *palA*, *B*, *C*, *E* or *F* mutation). *pacG-92* and -110, loss-of-function mutations in the putative structural gene for a non-repressible acid phosphatase (Caddick and Arst 1986), and *palcA-1* and -10, loss-of-function mutations in *palcA*, similarly do not suppress *palB-7* (and presumably therefore any other *palA*, *B*, *C*, *E* or *F* mutation). In all of these cases, phenotypes of double mutants are those expected from simple additivity.

Discussion

It is clear that *palA*, *B*, *C*, *E* and *F* and *pacC* mutations alter the regulation of expression of structural genes specifying extracellular enzymes and permeases by the pH of the growth medium. *palA*, *B*, *C*, *E* and *F* mutations mimic the effects of an acidic medium whereas *pacC* mutations mimic those of an alkaline medium. The first question is whether these mutations exert their primary effects on pH_{in} with regulatory effects as a secondary consequence in which case it must be assumed that the regulatory system actually monitors pH_{in}, given that pH_{in} is a function of extracellular pH (Fig. 4) or whether they act directly on the pH regulatory system. This question is answered by data in Fig. 4. If *palA*, *B*, *C*, *E* and *F* mutations mimic the effects of growth at acid pH as an indirect consequence of altered pH_{in}, they should result in a pH_{in} more acidic than that of the wild type. In fact, *palB-7* (and presumably all phenotypically indistinguishable *palA*, *B*, *C*, *E* and *F* mutations) results in a more alkaline pH_{in} (Fig. 4). If *pacC* mutations mimic the effects of growth at alkaline pH because their pH_{in} is altered, they should result in a pH_{in} more alkaline than that of the wild type. The opposite is true (Fig. 4). Therefore all of these mutations would appear to exert their primary effects on the pH regulatory mechanism and to have secondary effects on the pH homeostatic mechanism. It follows that the pH homeostatic mechanism itself must be subject to pH regulation. This is hardly surprising for an organism able to grow over a wide pH range, and it follows the precedent set by the regulation of the cytoplasmic pH-regulating, proton-translocating ATPase of *Streptococcus faecalis* by growth pH (Kobayashi et al. 1984; Kobayashi 1985). Thus *palA*, *B*, *C*, *E* and *F* mutants have an abnormally alkaline pH_{in} because their mimicry of acidic growth conditions leads them to over-react to protons. *pacC* mutations result in an abnormally acidic pH_{in} because their mimicry of alkaline growth conditions leads them to under-react to protons.

The next question is what relationship do the *palA*, *B*,

C, *E* and *F* and *pacC* genes have to each other. Several observations are relevant. Firstly, mutations in *palA*, *B*, *C*, *E* and *F* are apparently recessive in diploids (Dorn 1965a; this work) whereas *pacC-5* is partially dominant, *pacC-5*/*pacC*$^+$ diploids having about 75% of the intra- and extracellular acid phosphatase and phosphodiesterase of a *pacC*$^+$/*pacC*$^+$ diploid and about 120% of the alkaline phosphatase activity (Caddick, unpublished). It is possible that more extreme alleles (e.g. *pacC-14*) would show a greater degree of partial dominance. Secondly, non-leaky mutations in the five genes *palA*, *palB*, *palC*, *palE* and *palF* all have the same phenotype and, at least in the case of *palB-7 palC-4* double mutants, are non-additive. Although it is formally possible that a single entity would have five heterologous subunits, it seems much more likely that these genes participate in a metabolic pathway having, perhaps, five steps. Thirdly *pacC* mutations are epistatic to mutations in *palA*, *B*, *C*, *E* and *F* and to *palB-7* and *palC-4* in combination (other combinations not tested), indicating a more direct involvement for the *pacC* product than for that of *palA*, *B*, *C*, *E* and *F*.

A plausible model would be that *pacC* is a wide domain regulatory gene whose product directly mediates pH regulation whilst the products of *palA*, *B*, *C*, *E* and *F* participate in synthesis of an effector molecule which interacts with the *pacC* product. The partial dominance of *pacC* mutations is typical of that for mutations in a number of regulatory genes of *A. nidulans* (reviewed by Arst and Scazzocchio 1985). If these characterised *pacC* mutations are of a loss-of-function class, the basis for their epistasis to mutations in *palA*, *B*, *C*, *E* and *F* is clear: In the absence of a functional *pacC* product, the presence or absence of the cognate effector molecule would be of no consequence.

This model is strongly supported by the properties of some recently selected mutant *pacC* alleles (Caddick and Arst, unpublished). These mutations, provisionally designated *pacC**, share certain aspects to the phenotype of *palA*, *B*, *C*, *E* and *F* mutations and might form an altered function rather than loss-of-function class. Heterogeneous in phenotype, they result in any of the following: elevated acid phosphatase levels, reduced alkaline phosphatase levels, improved GABA utilisation at pH 6.5, suppression of *areA*^{r} mutations for GABA utilisation at pH 6.5, enhanced neomycin tolerance and reduced molybdate tolerance. The patterns with which different *pacC** alleles have these properties do not indicate that they form a hierarchy in which an allele can be classified as more extreme or less extreme. Non-hierarchical heterogeneity of allele phenotypes (Arst and Bailey 1977; Arst and Scazzocchio 1985) indicates direct involvement of the *pacC* product in the regulation of gene expression and shows that individual receptor sites for the *pacC* gene product, presumably adjacent to structural genes under its control, differ in structure.

If the above model be correct, the *pacC* product must control some activities (e.g. acid phosphatase PV, phosphate-repressible phosphodiesterase, GABA permease) in a positive manner and others (e.g. alkaline phosphatase PI) in a negative manner. The effector elaborated by the products of *palA*, *B*, *C*, *E* and *F* would antagonise the (positive and negative) action of the *pacC* product such that absence of the effector (or insensitivity to its presence in *pacC** strains) would enhance expression of structural genes subject to positive control by *pacC* and reduce expression of structural genes subject to negative control by *pacC*.

Whatever their precise roles, *palA*, *B*, *C*, *E* and *F* and *pacC* mutations are, we believe, the first mutations characterised as affecting the regulation of expression of structural genes specifying extracellular enzymes and permeases by the pH of the growth medium in any organism.

Acknowledgements. 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 and Claudio Scazzocchio for comments on the manuscript.

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Communicated by W. Gajewski

Received November 25, 1985 / January 7, 1986

Note added in proof

Growth of *Aspergillus nidulans* at pH 2.5 has been reported by C. Dijkema, R.P. Rijeken, H.C.M. Kester and J. Visser (FEMS Microbiol Lett 33:125–131 (1986)). Thus the pH range over which this organism will grow is even wider than stated in the text.