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pH regulation is a major determinant in expression of a fungal penicillin biosynthetic gene

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Transcription of the *ipnA* gene encoding isopenicillin N synthetase, an enzyme of secondary metabolism, is under the control of the pH regulatory system in the fungus *Aspergillus nidulans*. External alkaline pH or mutations in *pacC*, the wide domain regulatory gene which mediates pH regulation, override carbon regulation of *ipnA* transcript levels, resulting in elevation of the levels of this message in sucrose broth. Strains carrying these mutations, which mimic growth at alkaline pH, produce higher levels of penicillins when grown in sucrose broth compared with the wild type strain grown under carbon derepressing conditions. *ipnA* transcription is regulated by carbon (C) source, but extreme mutations in *creA* (the regulatory gene mediating carbon catabolite repression) only slightly increase repressed transcript levels. Precise deletion of the only *in vitro* CreA binding site present in a region of the *ipnA* promoter involved in carbon regulation has no effect on *ipnA* expression. The levels of *ipnA* transcript in broths with acetate or glycerol as principal C sources are inconsistent with direct or indirect *creA*-mediated transcriptional control of the gene. We conclude that a second, *creA*-independent mechanism of carbon repression controls expression of this gene. All derepressing C sources tested result in alkalinization of the growth media. In contrast, all repressing C sources result in external acidification. Neither acidic external pH nor *pal* mutations, mimicking the effects of growth at acid pH, prevent carbon derepression, providing strong support for independent regulatory mechanisms, one mediating carbon regulation (via thus far unidentified genes) and another mediating pH regulation (through the *pacC*-encoded transcriptional regulator). External pH measurements suggest that these two independent forms of regulation normally act in concert. We propose that external alkalinity represents a physiological signal which triggers penicillin biosynthesis.

Key words: carbon catabolite repression/fungi/pH regulation/secondary metabolism/transcriptional regulation

Introduction

In microbes, whereas primary metabolism is concerned with the exigencies of growth in the physical and chemical environment, secondary metabolism can be largely described as concerning survival and reproduction in the biological environment (although it might be triggered by physical or chemical factors). Secondary metabolism is frequently superfluous under laboratory conditions and therefore more elusive to study. It is commonly thought that nutrient exhaustion is a prerequisite for secondary metabolism. Here, however, we show that carbon derepression and/or external alkalinization (which can result from C source depletion) elevate penicillin titres and expression of at least one structural gene of penicillin biosynthesis.

Most of the time, micro-organisms respond to changes in environmental conditions by varying patterns of gene expression. In some cases, these variations involve adaptative responses of intracellular catabolic pathways. In others, they involve synthesis of exported molecules capable of modifying the environment. *Aspergillus nidulans* (Ascomycotina, Plectomycetes) displays remarkable physiological versatility. Of present relevance, it can use a wide variety of C sources (for review, see McCullough *et al.*, 1977). In addition to its metabolic versatility, this fungus (as well as many other filamentous ascomycetes) grows over a wide range of temperature, pH, osmolarity and ionic strength. Wild type strains of *A.nidulans* can grow in media over the pH range 2.5-10.5 (Rossi and Arst, 1990). Extracellular enzymes and permeases are not protected by the intracellular pH homeostatic system and their synthesis is controlled by external pH. This mode of regulation avoids the synthesis of, for example, secreted alkaline phosphatase in an acidic pH environment (Caddick *et al.*, 1986).

To our knowledge, *A.nidulans* is the only organism in which this pH regulatory circuit, which must be widespread in nature, has been genetically dissected (Caddick *et al.*, 1986). A major class of mutations in the *pacC* regulatory gene mimic the effect of growth at alkaline pH. For example, strains carrying these mutations secrete alkaline phosphatase at acidic pH and are repressed for synthesis of γ-aminobutyrate (GABA) permease (which has an acidic pH optimum). In contrast, mutations in *palA*, *palB*, *palC*, *palE* and *palF* have indistinguishable phenotypes and reproduce the effects of growth at acidic pH. For instance, they result in elevated levels of acid phosphatase and GABA permease at neutral or higher pH. Because *pacC* alleles show non-hierarchical heterogenity of mutant phenotypes, it has been proposed that the *pacC* product directly mediates regulation of expression of genes under its control. Indeed, the derived PacC sequence contains three putative C_2H_2 zinc-fingers (J.Tilburn and H.N.Arst, in preparation) supporting a role for PacC as a transcriptional regulator. In the original model (Caddick *et al.*, 1986; Shah *et al.*, 1991), PacC would act as a negative regulator of alkaline pH-specific genes at neutral or acidic pH. At alkaline pH an effector molecule, whose synthesis would be catalysed by the *pal* gene products, would prevent the negative action of PacC, thereby allowing

transcription of alkaline pH-specific genes under its control. In this model, the class of mutations in *pacC* which mimic the effect of growth at alkaline pH represents the loss-of-function class.

However, recent analysis of transcriptional regulation of *pacC* itself as well as sequence analysis of a range of *pacC* mutations indicates that this major class of mutations mimicking alkalinity, all of which lie in the 3′ portion of the ORF and considerably downstream of the 'zinc finger' encoding region, actually comprises gain-of-function alleles (J.Tilburn, S.Sarkar, D.Widdick and H.N.Arst, unpublished). This supports a model in which PacC is a transcriptional activator at alkaline pH and the *pal* gene products catalyse the synthesis of an effector which potentiates the action of PacC (J.Tilburn and H.N.Arst, in preparation). Thus, *pacC* mutations which mimic the effects of growth at alkaline pH obviate the requirement for the effector molecule.

The domain of action of the pH regulatory system extends to small exported molecules, such as penicillins (Shah *et al.*, 1991), which are powerful antimicrobial secondary metabolites. Here we describe analysis of the expression pattern of the *ipnA* gene [encoding isopenicillin N synthetase, a key biosynthetic enzyme in the penicillin pathway (for a review see Luengo and Peñalva, 1993)] at different pH values or in mutant *pacC* backgrounds. This analysis strongly indicates that penicillin production is higher at alkaline pH because the PacC regulatory protein positively modulates transcription of at least this penicillin structural gene.

Several Plectomycetes, including *A.nidulans,* synthesize penicillins as secondary metabolites. Penicillin biosynthesis is regulated at the level of transcription of penicillin structural genes (Peñalva *et al.*, 1989; MacCabe *et al.*, 1990; Espeso and Peñalva, 1992; Pérez-Esteban *et al.*, 1993). Expression of the *ipnA* gene is under carbon regulation (Espeso and Peñalva, 1992). The levels of its message (as well as of extracellular penicillin accumulation) are repressed when the growth medium contains a preferred C source (such as sucrose or glucose). Carbon catabolite repression of primary metabolism gene expression is exerted through the action of the transcriptional repressor encoded by the *creA* gene (Arst and MacDonald, 1975; Bailey and Arst, 1975; Dowzer and Kelly, 1991). All tested mutant *creA* alleles, including the extreme *creA*^{d}-30 (Arst *et al.*, 1990), only slightly derepress *ipnA* mRNA levels. Although this relative lack of effect might be due to differential effects of loss-of-function *creA* mutations on expression of different structural genes (no effective allele having yet been found), this suggests that a second, *creA*-independent mechanism of carbon repression might be operating on penicillin biosynthesis (Espeso and Peñalva, 1992). We show here that such a second mechanism of carbon repression must control our model penicillin structural gene.

Finally, our results indicate that carbon repression and pH regulation of *ipnA* expression are mediated through different mechanisms. Normally, both regulatory circuits act in concert, as all *ipnA*-derepressing C sources result in early external alkalinization. However, this carbon regulation is overcome by growth at alkaline pH (or by the above-mentioned *pacC* mutations), indicating the overriding importance of pH regulation to penicillin biosynthesis.

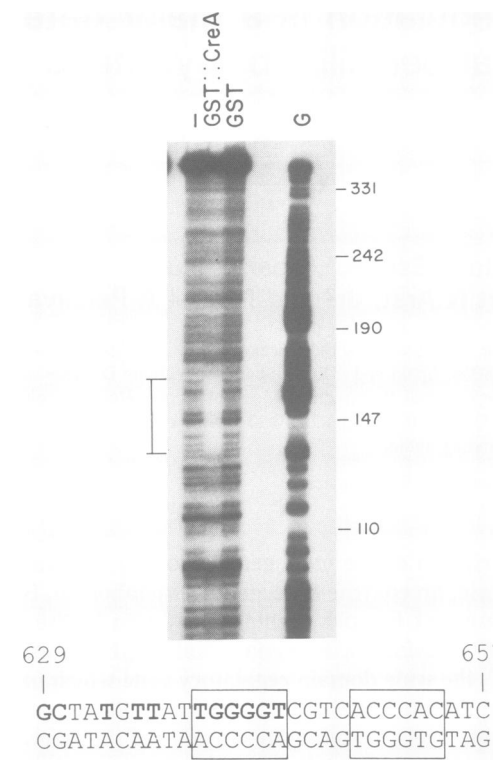

Fig. 1. *In vitro* footprint of CreA in a region of the *ipnA* promoter which is physiologically relevant to carbon repression. A fragment containing the only binding site for a purified chimeric GST::CreA(35−240) polypeptide in the region of the *ipnA* promoter which mediates carbon repression was terminally labelled at one end and purified. The GST::CreA(35−240) fusion (Kulmburg *et al.*, 1993) carries amino acids 35−240 of the transcriptional repressor CreA. This region includes a functional DNA binding domain (two C_2H_2 zinc fingers). The fragment was incubated in the presence of either 5 μg of this fusion protein (GST::CreA), an equal amount of purified GST polypeptide (GST) or the buffer in which proteins were stored (−) and treated with DNase I. Excess GST::CreA(35−240) protein was used to maximize the likelihood of detecting CreA binding sites. Digested complexes were resolved in 6% polyacrylamide−6 M urea gels, alongside a 'G' Maxam and Gilbert reaction made on the same fragment as size standard. A prominent footprint (also confirmed using the other strand, not shown) is found, corresponding to positions 629−657 from a *Xho*I site located 2005 bp upstream of the *ipnA* ATG (see Pérez-Esteban *et al.*, 1993). The nucleotide sequence protected from DNase I digestion is shown below. This sequence contains two nearly consensus CreA binding sites (boxed, see also text). Shown in bold are the nucleotides at and around the upstream site which are identical (in the G-rich strand) to those present in the sequence protected by *S.cerevisiae* MIG1 protein in the *GAL1* promoter (Nehlin *et al.*, 1991). MIG1 and CreA are functional homologues and have almost identical DNA binding domains (Dowzer and Kelly, 1991).

Results

Lack of evidence for functional relevance in vivo of in vitro CreA binding to the ipnA promoter

We have previously shown that *creA*^{d} mutations only slightly increase *ipnA* transcript levels under repressing conditions (Espeso and Peñalva, 1992). This result does not rigorously rule out a role for *creA* in regulating *ipnA*, as the particular alleles tested might affect *ipnA* expression much less than expression of other genes. For a more direct approach, we have tested *in vitro* the binding of a purified chimeric protein containing glutathione S-transferase (GST)

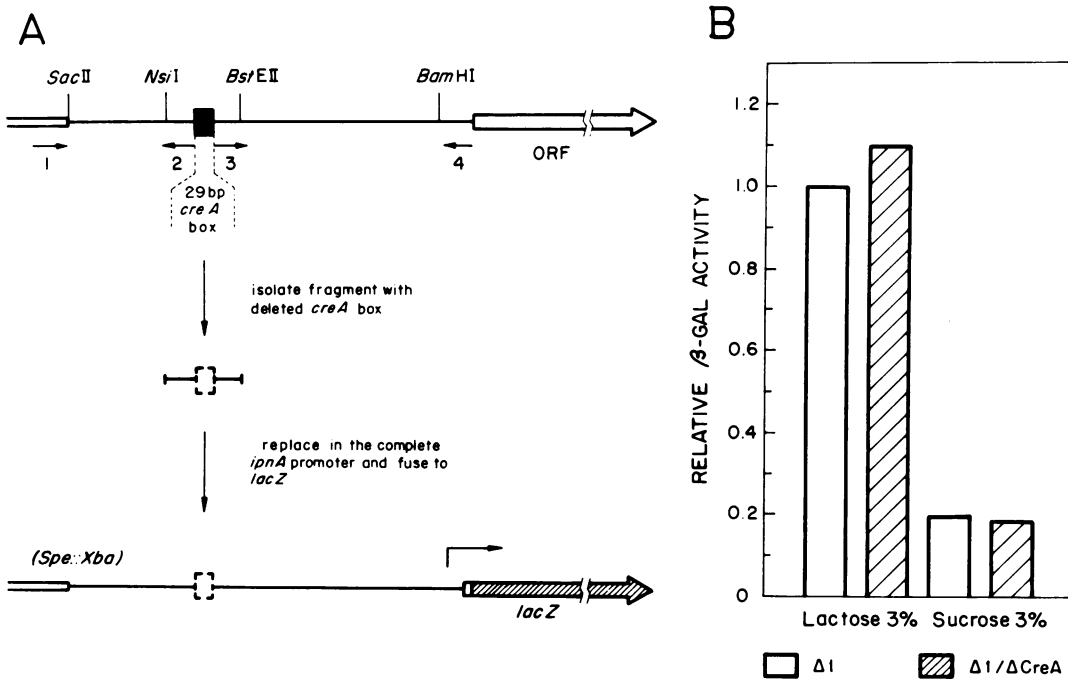

Fig. 2. *In vivo* effect of the precise deletion of the CreA binding site mapped by DNase I footprinting. (**A**) The scheme summarizes how a complete *ipnA* promoter lacking the 29 bp protected by GST::CreA(35−240) (see Figure 1) was reconstructed by ligation of two fragments obtained after PCR amplification with primers 1 and 2 or 3 and 4, respectively, using cloned *ipnA* promoter DNA as template. The resulting promoter DNA, containing the deletion, was restricted with *Nsi*I and *Bst*EII to isolate the 0.3 kb fragment carrying the mutation, which was completely sequenced to confirm that no mutations were generated by PCR amplification. The equivalent *Nsi*I−*Bst*EII fragment in the *ipnA'::'lacZ* construct pBSΔ1 (Pérez-Esteban *et al.*, 1993), which contains the complete *ipnA* promoter (at least in terms of carbon repression), was replaced by the fragment containing the deletion. The recombinant construct was transformed into *A.nidulans* and a transformant carrying a single copy integration at *argB* was identified and purified. It was named Δ1/ΔCreA. (**B**) Cultures of this strain and of strain Δ1 (single copy integration of pBSΔ1, i.e. the wild type promoter, at *argB*, otherwise isogenic) were grown in either 3% lactose or 3% sucrose and used to prepare protein extracts in which β-galactosidase activity was assayed. Duplicate assays obtained from independent cultures agreed within 10%.

and the CreA DNA binding domain [GST::CreA(35−240), Kulmburg *et al.*, 1993] to the *ipnA* upstream region and detected several CreA binding sites (E.Espeso and M.A.Peñalva, in preparation). The abundance of *in vitro* CreA binding sites reflects most likely the high G + C content of the *ipnA* promoter region, as all of these sites are very close to the CreA consensus G/CPyGGGG determined by Kulmburg *et al.* (1993). However, functional analysis of the *ipnA* promoter has shown that a *cis*-acting region crucial to sucrose repression maps between −1334 and −966 relative to the transcriptional start site of the gene (Pérez-Esteban *et al.*, 1993). A single CreA binding site can be detected in this region. Its DNase I footprint is shown in Figure 1. A 29 nt region is protected. The first 16 nt, which include a nearly canonical CreA binding site, have 11 identities with the *MIG1* footprint in the *Saccharomyces cerevisiae GAL1* promoter (*MIG1* is the yeast homologue of *creA*). The derived sequences of CreA and MIG1 proteins are almost identical in the DNA binding region (Dowzer and Kelly, 1991). The fact that CreA protects 29 nt from DNase I digestion suggests that the GST::CreA(35−240) protein is binding as a dimer, not unexpectedly as the GST moiety contains a dimerization domain. Consistent with this interpretation, a second nearly canonical CreA binding site can be recognized on the other strand (GTGGGT). These results suggest that if *ipnA* transcription be under direct *creA* control, such a control would be mediated through this site, although a role for other CreA binding sites cannot be definitively ruled out (Pérez-Esteban *et al.*, 1993).

We have used reverse genetics to demonstrate the lack of function *in vivo* of this binding site. An *ipnA'::'lacZ* fusion containing the promoter region lacking precisely the 29 nt protected region was constructed using the strategy shown in Figure 2A. A strain containing a single copy integration of this construct at the *argB* locus was characterized by Southern analysis. Mycelia of this strain and of a control strain carrying one copy of the parental *ipnA'::'lacZ* fusion integrated at the same chromosomal location were grown in penicillin production broth (PPB) containing either sucrose or lactose as principal C source (carbon repressing or derepressing conditions, respectively). β-galactosidase assays using mycelial protein extracts (Figure 2B) show that deletion of these 29 bp does not affect sucrose repression of the chimeric gene, suggesting that this *in vitro* CreA binding site plays no role in carbon regulation. It follows that carbon regulation of *ipnA* expression probably does not involve direct binding of the *creA* product to the promoter region.

ipnA transcript levels in broths containing glycerol or acetate as principal C sources are independent of creA control

Results with C2 and C3 compounds as C sources provide further evidence against a role for CreA in the control of

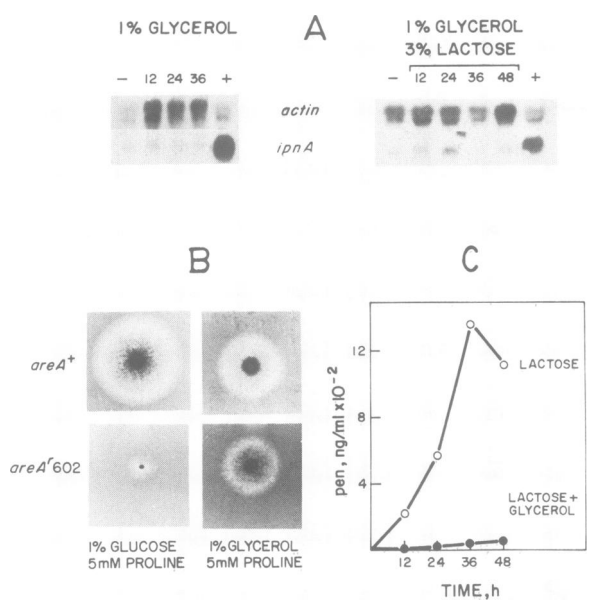

Fig. 3. Glycerol represses penicillin biosynthesis as well as *ipnA* transcription. (**A**) Northern analysis of total RNAs isolated from mycelia grown in penicillin production broth containing either 1% glycerol or 1% glycerol + 3% lactose as C source. The time (h) after inoculation in which mycelia were harvested is indicated. '+' and '−' symbols indicate RNA extracted from mycelia grown in 3% lactose or 3% sucrose, respectively, after 12 h of growth and represent positive and negative controls. These RNA samples have been included in most Northerns to allow comparison between different figures. The probes were specific for actin (loading control) and *ipnA* messages. (**B**) *A.nidulans* strains having the relevant genotypes indicated were inoculated in minimal medium with C and N sources as indicated. In the presence of a repressing carbon source such as 1% (w/v) D-glucose, *areA*^{r} loss-of-function mutations such as *areA*^{r}-602 prevent utilization of L-proline as N source (Arst and Cove, 1973). In contrast, C sources which are derepressing *via* the *creA* circuit [such as 1% (v/v) glycerol] allow *areA*^{r}-602 strains to utilize proline as N (Arst and Cove, 1973). (**C**) Extracellular penicillin accumulation in the cultures used in panel A; pen indicates penicillin.

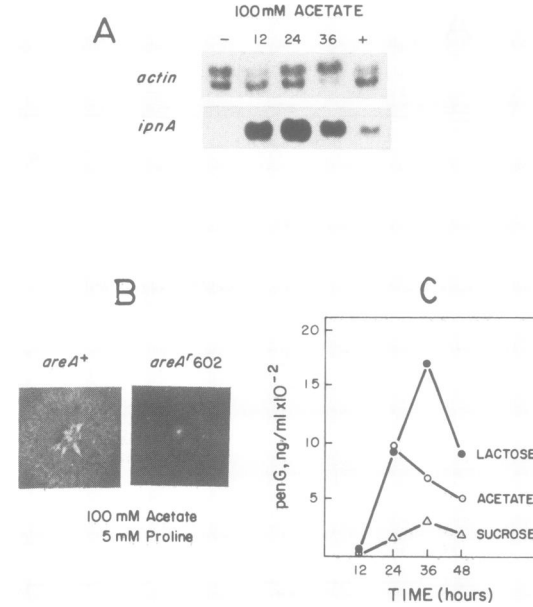

Fig. 4. Acetate is not a repressing C source for *ipnA* transcription although it is repressing through the *creA* circuit. Panels **A**, **B** and **C** are as in Figure 3, except for the C sources used. Refer to Figure 3B for the glucose control for panel B.

ipnA transcription. Glycerol is a typical derepressing C source in the *creA*-mediated circuit of carbon catabolite repression. It eliminates the strict requirement for the *areA* gene product for use of L-proline as sole nitrogen (N) source (Arst and Cove, 1973; see Figure 3B) and allows complete derepression of *alcA* expression (Arst and Bailey, 1977). Nevertheless, *A.nidulans* mycelia grown in PPB containing glycerol as principal C source export very little penicillin (data not shown) and contain almost undetectable levels of the *ipnA* message (Figure 3A). Moreover, inclusion of glycerol in lactose−PPB almost completely abolishes penicillin biosynthesis (Figure 3C) and causes repression of the otherwise derepressed levels of the *ipnA* message (Figure 3A). Control of *ipnA* transcription by *creA* is difficult to reconcile with repression caused by glycerol.

Acetate is a typical repressing C source. It does not allow the use of L-proline as sole N source in the absence of a functional *areA* allele (Figure 4B; Arst and Cove, 1973). Nevertheless, substantial levels of penicillins are made by *A.nidulans* in PPB containing 100 mM acetate as principal C source (Figure 4C), in spite of the fact that acetate probably competes with phenylacetate, the lateral side chain precursor, for CoA thioester formation (Martínez-Blanco *et al.*, 1992). Furthermore, acetate results in even greater derepression of *ipnA* message levels than lactose (Figure 4A), a typical derepressing C source for expression of both genes under *creA* control and *ipnA*. Derepression resulting from acetate utilization is hard to reconcile with *creA* control of *ipnA* message levels. We therefore conclude that carbon repression of *ipnA* expression is not mediated, directly or indirectly, through the *creA* product.

Alkaline pH bypasses carbon regulation of ipnA gene expression

Previous work (Shah *et al.*, 1991) showed that penicillin biosynthesis is under pH regulation, being greatest at alkaline pH. We therefore considered the possibility that external alkaline pH could bypass sucrose repression of penicillin structural gene expression. The experiment in Figure 5 indicates that this is indeed the case. Inclusion of 100 mM Na_2HPO_4 in sucrose broth results in an initial pH of 8.0. This broth was inoculated with wild type *A.nidulans* conidiospores and a control culture was established in standard sucrose−PPB in the absence of this high buffering capacity. Northern analysis of *ipnA* message levels in mycelial samples taken at different times shows that growth of the fungus in sucrose−PPB buffered at alkaline pH causes complete derepression at both tested times of the growth cycle. However, despite the high buffering capacity, external pH nevertheless decreased, showing that medium acidification (which accompanies sucrose catabolism, *vide infra*) still occurs. Inclusion of 200 or 300 mM Na^+ (as Na_2SO_4 or NaCl) in sucrose−PPB did not substantially change carbon-repressed *ipnA* message levels (data not shown). This rules out the possibility that changes in steady state transcript levels are due to the increase in external [Na^+] rather than to pH. Buffering at alkaline pH with Tris−HCl increases penicillin titres in glucose media (Shah *et al.*, 1991), suggesting that these increases are not caused by phosphate ions. This was rigorously confirmed using transfer experiments (*vide infra*). We conclude that alkaline external pH *per se* derepresses *ipnA* transcription. Moreover,

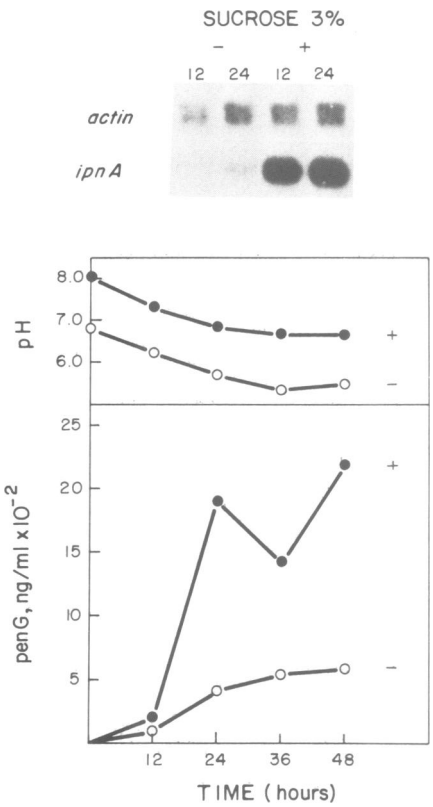

Fig. 5. Growth at (nearly) alkaline pH results in bypass of carbon repression. *A.nidulans* cultures were grown in 3% sucrose broth containing (+) or not (−) 100 mM Na_2HPO_4. Samples of mycelia were harvested at the times indicated and used to isolate RNA which was analysed by Northerns as in Figure 3. The profiles of external pH for these cultures, together with time-courses of penicillin production in each culture are indicated.

high external pH not only derepressed *ipnA* gene expression but also resulted in derepressed levels of penicillin production, suggesting that external alkaline pH is or provokes a physiological signal triggering penicillin biosynthesis in *A.nidulans*.

A transfer experiment confirmed the above conclusions: *A.nidulans* was inoculated in 3% sucrose−PPB (initial pH 6.8 with 10 mM sodium phosphate buffer). After 12 h growth at 37°C (after which the external pH was 5.5), equal portions of mycelia were transferred to flasks containing fresh sucrose−PPB with 100 mM NaH_2PO_4 and 100 mM MES. The broth in each flask (otherwise identical to that of the remaining flasks) was adjusted with different amounts of NaOH to a pH within the range 5.0−8.0 (see Materials and methods). .These transferred cultures were further incubated for 6 h at 37°C (during which pH variation was negligible). Figure 6 shows that when extracellular penicillin accumulation (corrected for mycelial growth) was measured in the different cultures, the higher the pH of the broth, the greater the amount of antibiotic. Controls (not shown) indicated that penicillin stability in sucrose−PPB is very similar at pH 5.0 or 8.0. As all the cultures contained identical concentrations of MES and phosphate ions (100 mM), this experiment, together with that described above, shows that alkaline pH is the factor derepressing penicillin production in 3% sucrose broth, eliminating high phosphate concentrations as the agent able to bypass carbon repression. Figure 6 also shows a Northern analysis of *ipnA* message

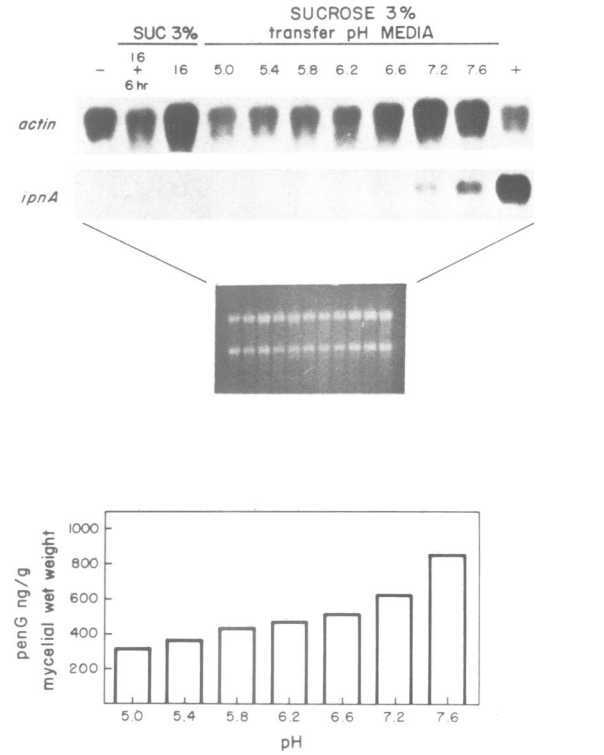

Fig. 6. Transfer of mycelia grown under carbon repressing conditions to buffered sucrose broth shows that alkaline pH overcomes sucrose repression. *A.nidulans* was grown in sucrose−penicillin production broth for 16 h at 37°C (external pH at that time, 5.5) and harvested by filtration. Mycelia were divided in identical portions (in wet weight) which were transferred to different flasks, all containing fresh 3% sucrose−penicillin production broth plus 100 mM MES and 100 mM NaH_2PO_4, adjusted with NaOH to the pH values indicated. Because of pH adjustment, the culture at pH 7.6 differs from that at pH 5.0 (no NaOH addition) by the presence of 172 mM additional Na^+. Inclusion of up to 300 mM Na^+ in pH 5 sucrose−PPB (containing 100 mM NaH_2PO_4) did not overcome carbon repression of *ipnA* transcript levels (see text for details). These transferred cultures were further incubated for 6 h at 37°C. After this time, broth samples were taken to measure penicillin and the mycelia in each flask were harvested, weighed and used for RNA isolation. pH variations in these transferred cultures were negligible. The upper part of the figure shows a Northern blot of these RNA samples. '−' and '+' controls are as in Figure 3. '16 + 6 h' represents an RNA sample from mycelia transferred to 3% sucrose−penicillin production broth without MES and phosphate, initial pH 6.8 and '16' is an RNA sample from 16 h old mycelia used for the transfer. The blot was hybridized with *ipnA*- and actin-specific probes, as indicated. Ethidium bromide staining of the gel is also included. The lower part shows extracellular penicillin accumulation in cultures at the different pH values (indicated below). Samples were taken 6 h after the transfer.

levels in these cultures. Controls showing the steady state levels of *ipnA* mRNA in the original cultures at the time of transfer (16 h) or in mycelia transferred to standard PPB−sucrose broth are included for comparison, together with a sample corresponding to a culture grown in a derepressing C source. Transcript levels were almost undetectable (even lower than in the original culture) after transfer to broths between pH 5 and 6.2, but above this range, transcript levels rise and are at least partially derepressed after 6 h at the final pH, despite the presence of 3% sucrose. Moreover, above pH 6.2, the higher the external pH after the transfer, the greater the levels of *ipnA* message detected. This experiment strongly implicates external alkaline pH in the bypass of sucrose repression. Therefore, data in Figures 5 and 6 show that an external alkaline pH is sufficient to give

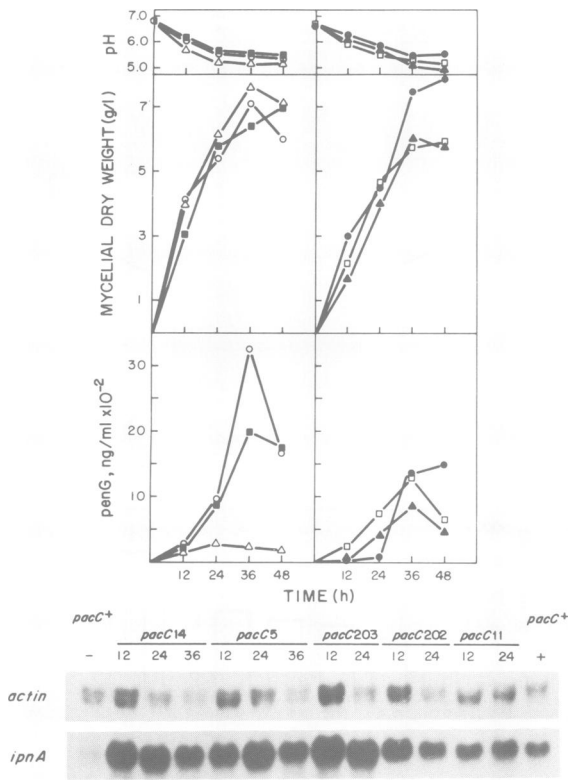

Fig. 7. Effect of different gain-of-function mutations in *pacC* on sucrose-repressed *ipnA* transcript levels. Mycelia of strains carrying the different *pacC* alleles indicated were obtained in penicillin production broth with 3% sucrose at the times indicated. Samples were taken at different times to measure external pH, mycelial growth and penicillin accumulation in the cultures. Mycelia were used for RNA isolation. RNA was analysed by Northern blot hybridization. Symbols and numbers on top of each track are as in Figure 3. Graph symbols are as follows: △, *pacC*$^+$; ▲, *pacC*11; ■, *pacC*5; □, *pacC*202; ○, *pacC*14; ●, *pacC*203.

rise to considerable transcriptional derepression of at least one penicillin structural gene and of penicillin biosynthesis.

Mutations in pacC bypass carbon regulation of ipnA transcript levels

If alkaline pH were directly responsible for elevating *ipnA* expression in carbon repressing media, it would be predicted that mutations in the wide domain regulatory gene *pacC* which mimic the effect of growth at alkaline pH should overcome carbon regulation. Figure 7 shows penicillin fermentations (in the presence of 3% sucrose) with strains carrying different mutant *pacC* alleles. In agreement with previous results (Shah *et al.*, 1991) using *Aspergillus* complete medium (Cove, 1966), which contains 1% glucose as C source, all strains carrying a *pacC* mutation gave rise to higher penicillin titres than the wild type, despite a lack of effect on external pH or growth yields (Figure 7). This figure also illustrates that these *pacC* mutations cause derepression of steady state levels of the *ipnA* message (despite external acidic pH resulting from sucrose utilization). This result is fully consistent with the proposed role of PacC as a transcriptional regulator (Caddick *et al.*, 1986; J.Tilburn and H.N.Arst, unpublished) and indicates that these *pacC* mutations elevate penicillin biosynthesis by activating expression of at least one penicillin structural gene even in the presence of 3% sucrose. In addition, this further confirms that the pH regulatory circuit (and not phosphate or sodium ions) effects changes of *ipnA* gene expression.

Acidic pH or pal mutations do not prevent carbon regulation of ipnA transcript levels

A simple interpretation of the results described above would be that carbon repression is mediated through pH regulation, which would be consistent with the influence of the different C sources on external pH (*vide infra*). So, derepressing C sources would elevate *ipnA* transcription because they cause external alkalinization, whereas repressing C sources would reduce *ipnA* transcript levels by acidification (*vide infra*). It would therefore follow that buffering of lactose−PPB at acidic values should override carbon derepression. Figure 8A shows that continous growth of the fungus in lactose broth buffered (two different capacities tested) at acidic pH did not cause a substantial reduction in carbon-derepressed *ipnA* transcript levels. This lack of effect was confirmed with 0.1% D-glucose (derepressing concentration) as principal C source (data not shown). Moreover, transfer of lactose-grown (i.e. derepressing for carbon, external pH alkaline) mycelia to fresh lactose broth buffered at acidic pH (down to pH 5.4, Figure 8B, i.e. reciprocal experiment to that shown in Figure 6) did not result in a significant reduction in derepressed transcript levels. We conclude that acidic pH does not override carbon derepressed transcript levels.

Additionally, if the above interpretation (i.e., carbon repression mediated through pH regulation) were true, mutations in *pal* genes, which mimic the effect of growth at acidic pH (Caddick *et al.*, 1986), should prevent *ipnA* derepression in lactose medium. We have tested strains carrying putative loss-of-function mutations *palA*1, *palB*7 and *palF*15, representing three different genes, in lactose−PPB, and found that *ipnA* transcript levels are essentially derepressed (Figure 9). We conclude that neither external acidic pH nor loss of *pal* gene function prevents derepression caused by growth in lactose. These results strongly indicate that the simple interpretation in which pH changes account for C source effects is not tenable.

The effect of different carbon sources on external pH

We have shown that the effect of growth in a repressing C source on *ipnA* transcription can be overcome by external alkaline pH or *pacC* mutations, whereas the effect of growth in a derepressing C source cannot be prevented by external acid pH or *pal* mutations. This result is consistent with independent controls mediating carbon and pH regulation, a conclusion supported by further arguments (see Discussion). These controls normally act in concert (at least under standard conditions). Growth of *A.nidulans* in PPB (initial pH, 6.8) containing 3% lactose, 0.1% D-glucose, 0.1% D-fructose, 1% L-arabinose or 100 mM acetate (Na^+) as principal C source resulted in alkalinization of external pH (Figure 10). All of these compounds behave as derepressing C sources with respect to *ipnA* transcription (Espeso and Peñalva, 1992; this work). In contast, growth in 3% sucrose, 1% D-glucose or 1% glycerol resulted in media acidification (Figure 10). These three compounds are repressing C sources. In other words, carbon limitation, either by using less favourable C sources or by reducing the concentration of favourable C sources, results in external alkalinization, whereas availability of a sufficient, favourable

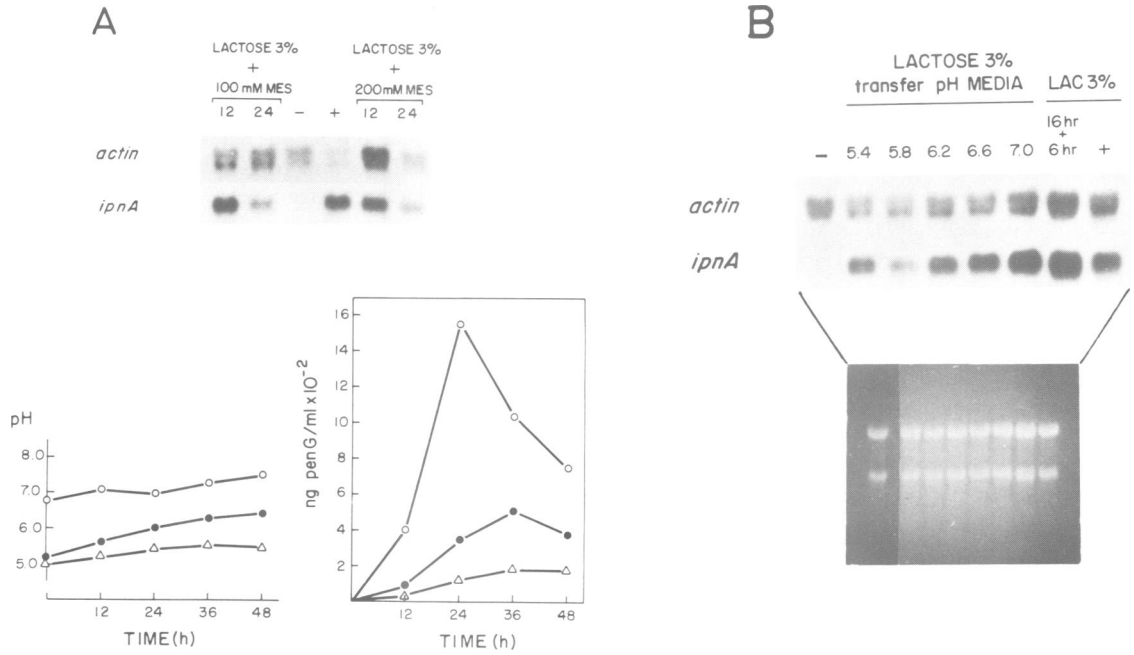

Fig. 8. Acidic pH does not prevent elevation of *ipnA* transcript levels resulting from growth in a derepressing C source. (**A**) Northern analysis of *ipnA* message levels in RNA samples from 12 h- and 24 h-old mycelia grown in lactose broth (carbon derepressing conditions). '+' and '−' samples are as in Figure 3. pH and penicillin accumulation profiles corresponding to the different cultures are shown below. Symbols are as follows: ○, lactose broth (initial pH, 6.8); ●, lactose broth, 100 mM MES (initial pH, 5.2); △, 200 mM MES (initial pH, 5.0). (**B**) Northern analysis of *ipnA* transcript in RNA samples isolated from mycelia grown for 16 h in lactose−PPB (external pH at that time, 7.2) and transferred to fresh lactose−PPB or to fresh lactose broth containing 100 mM MES and 100 mM NaH_2PO_4 adjusted (as in Figure 6) to the indicated pH values. '+' and '−' controls are as in Figure 3.

C source results in external acidification. Thus, carbon and pH regulation normally act in concert. As external acidic pH does not prevent carbon derepression, the acidification resulting from glycerol metabolism (Figure 10) cannot explain the repressing effect of glycerol and establishes glycerol as a repressing C source with respect to *ipnA* transcription.

Discussion

We have investigated the pattern of expression of a secondary metabolism gene (encoding isopenicillin N synthetase) in *A.nidulans*, particularly in response to environmental changes in C source availability or external pH. Our results provide experimental support for (normally) concerted (although mechanistically independent) actions of carbon and pH regulation on *ipnA* gene transcription.

Expression of the *ipnA* gene is under the control of the pH regulatory circuit. Mycelia grown in sucrose at alkaline pH or transferred from low buffer sucrose broth (external pH acidic) to alkaline pH contain derepressed levels of *ipnA* transcript. Therefore, external alkaline pH bypasses carbon repression. Controls indicate a genuine effect of the pH, rather than of the buffering ions. Moreover, mutations in the *pacC* regulatory gene which mimic growth at alkaline pH activate *ipnA* transcription in broths with sucrose, a repressing C source in which growth of the fungus causes strong acidification. This strongly suggests that elevation of *ipnA* transcript levels in alkaline sucrose broth is *pacC*-mediated. The occurrence of transcript elevation at acidic external pH in *pacC* mutants probably eliminates the possibility that carbon limitation leading to derepression derives from dissipation of a proton gradient driving force for sugar transporters through external alkalinization.

ipnA expression is under carbon regulation (Espeso and Peñalva, 1992). Carbon catabolite repression of primary metabolism gene expression involves the wide domain regulatory gene *creA* (Arst and Cove, 1973; Bailey and Arst, 1975; Arst *et al.*, 1990), which encodes a transcriptional repressor (Dowzer and Kelly, 1991; Kulmburg *et al.*, 1993). Our results show that a second, *creA*-independent mechanism of carbon regulation must exist, possibly controlling secondary metabolism genes. This conclusion is supported by the following arguments. (i) Even extreme loss-of-function mutations in *creA* only slightly derepress *ipnA* message levels (Espeso and Peñalva, 1992). This derepression is explicable in terms of pH regulation (*vide infra*). (ii) Pérez-Esteban *et al.* (1993) defined a *cis*-acting region in the *ipnA* promoter which is physiologically implicated in carbon regulation. This region contains a single *in vitro* CreA binding site, whose precise deletion does not affect *in vivo* expression in repressing or derepressing conditions. (iii) Glycerol and acetate, which are derepressing and repressing C sources, respectively, in the *creA*-dependent carbon regulation of primary metabolism genes, behave exactly opposite to what would be expected from *creA* control. This result precludes even an indirect effect of *creA* (mediated through a subordinate regulatory protein under CreA control). Possibly in keeping with its role as a repressing C source in the *creA*-independent carbon regulation system, glycerol also exerts a repressing effect on synthesis of the phenylacetate transport system, an accesory activity of the penicillin pathway (Fernández-Cañón *et al.*, 1989).

The concerted action of pH and carbon regulation

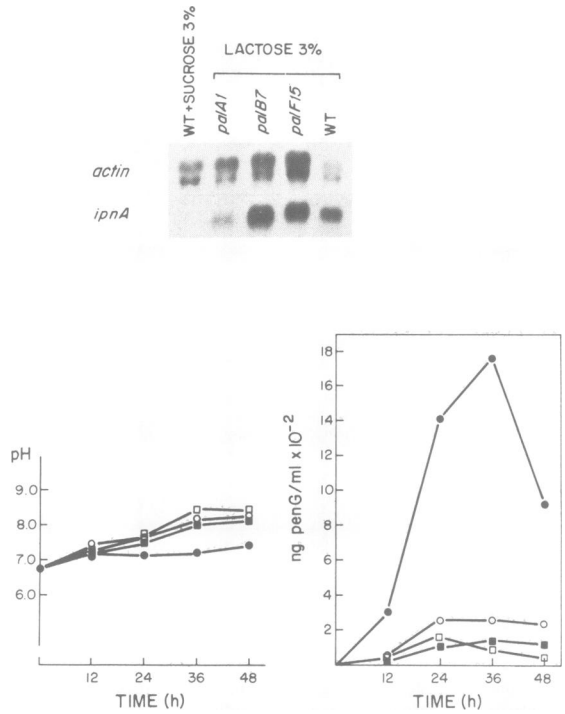

Fig. 9. Mutations in *pal* genes do not prevent derepression of *ipnA* transcript levels resulting from growth in lactose. The upper part shows a Northern analysis of *ipnA* transcript levels in RNA isolated from mycelia of strains carrying indicated *pal* mutations grown for 12 h in lactose–PPB. Controls showing carbon-repressed or derepressed *ipnA* message levels present in wild type mycelia after growth in sucrose- or lactose–PPB, respectively, are included for comparison. Because *pal* mutants grow increasingly poorly the higher the pH and accumulate supressor mutations, it is not feasible to obtain sufficiently good RNA preparations from 24 h (or longer) cultures, because of the rise in pH which accompanies lactose utilization. The profiles of pH and penicillin accumulation in the different lactose cultures are also shown. Symbols are as follows: ●, wild type (WT); ○, *palA*1; □, *palB7*; ■, *palF*15.

envisaged here (*vide infra*) might explain the slight effect (Espeso and Peñalva, 1992) of extreme loss-of-function mutations in *creA* on steady state levels of *ipnA* message, despite the lack of *creA* control of *ipnA* expression. These pleiotropic mutations reduce external pH acidification in 1% glucose (E.Espeso and M.A.Peñalva, unpublished). The slightly elevated message levels might therefore be effected through the pH regulatory circuit.

Measurements of external pH during growth of *A.nidulans* in PPB with different C sources allow their classification in two groups: all (tested) derepressing C sources alkalinize external pH, whereas all repressing C sources acidify it. This remarkable correlation can be explained in two ways: either carbon repression is mediated through pH regulation and *ipnA* expression would be derepressed only if external pH is alkaline (artificially or through the use of a non-repressing C source), or carbon repression and pH regulation act in concert, although through different mechanisms. The following arguments strongly favour the second possibility. (i) If carbon repression were mediated through pH regulation, external acidic pH or mutations in any of the *pal* genes [which mimic growth at acidic pH probably through inability to make an effector molecule necessary for the action of the *pacC* product (Caddick *et al.*, 1986; Shah *et al.*, 1991)] would be expected to prevent derepression by growth in 3% lactose. Our experiments show no drastic effects of external acid pH or *pal* mutations on carbon derepressed *ipnA* levels. (ii) The *cis*-acting region physiologically involved in carbon regulation of *ipnA* expression is subject to negative control (Pérez-Esteban *et al.*, 1993), whereas pH regulation probably requires PacC positive action. (iii) A fusion polypeptide containing the PacC DNA-binding domain does not bind *in vitro* to the *cis*-acting region which mediates carbon repression but does bind specifically to a different, functionally relevant region of the *ipnA* promoter (our unpublished results).

Putative gain-of-function mutations in *pacC* or external

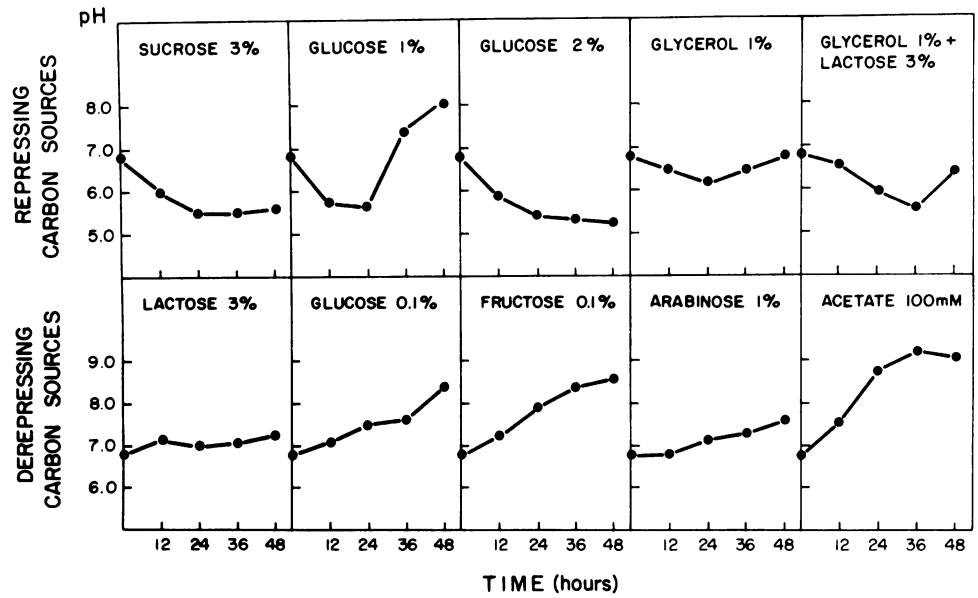

Fig. 10. Profiles of external pH in PPB with different C sources. Cultures of *A.nidulans* were grown at 37°C in penicillin production broth containing indicated C sources. At different times, samples, filtered through Miracloth to remove mycelia, were used to measure external pH. 'Repressing' and 'derepressing' refer to the two categories of C sources classified by effect on *ipnA* transcript levels (see Espeso and Peñalva, 1992, this work).

alkaline pH cause penicillin overproduction (Shah *et al.*, 1991), even under strongly carbon repressing conditions (this work). Mutations in any of the *pal* genes or external acidic pH impair penicillin production (Shah *et al.*, 1991), even under carbon derepressing conditions (this work), despite lack of a significant effect on *ipnA* message levels. There must therefore be at least one target for pH regulation in addition to the *ipnA* gene.

Why should pH and carbon regulation act in a concerted manner? It is likely that the use of less favourable C sources raises internal pH, as demonstrated in *Saccharomyces cerevisiae*, where a shift from glucose to ethanol results in an internal alkalinization of 0.4 pH units (den Hollander *et al.*, 1981). We speculate that C source limitation results in amino acid catabolism with subsequent (detectable by odour!) release of ammonia raising internal pH. This interpretation provides a physiological explanation for the concerted action of carbon and pH regulation. However, the fact that pH regulation overrides carbon repression suggests that in alkaline environments, penicillin biosynthesis is advantageous to the fungus, even if the carbon source is not limiting. Similarly, limiting C sources allow derepression of *ipnA* expression at any external pH. In other words, carbon derepression of *ipnA* transcription bypasses the requirement for PacC. Alternatively acting wide domain transcriptional regulators, one negative- the other positive-acting, are not without precedent in *A.nidulans*, and have been studied in detail in the proline catabolism gene cluster (see Sophianopoulou *et al.*, 1993, and references therein). Experiments to elucidate this dual factor regulation at the penicillin gene cluster are in progress.

The influence of external pH on regulation of peptide antibiotic biosynthesis might not be limited in occurrence to fungi. Haavik (1974) described how mildly alkaline pH favours bacitracin production by *Bacillus licheniformis* and inclusion of the neutralizing agent $CaCO_3$ prevents the inhibitory effect of glucose metabolism-derived acidification.

Materials and methods

Strains, media and growth of A.nidulans

The genotype of *A.nidulans* strains used is as follows (for gene nomenclature see Clutterbuck, 1990): *biA*1 (wild type); *pacC*5, *pabaA*1; *pacC*14, *biA*1; *pacC*11, *pantoB*100 ; *pacC*202, *pabaA*1; *pacC*203, *pabaA*1, *pantoB*100, *fwA*1; *palA*1, *wA*3, *biA*1; *palB*7, *pabaA*1, *chaA*1; *palF*15, *pantoB*100, *yA*2. All strains carrying relevant mutations in the pH regulatory circuit were from the collection of H.N.Arst, as was a strain carrying the loss-of-function allele *areA*^{r}-602 (Al Taho *et al.*, 1984) in a *biA*1 background. A strain of genotype *biA*1, *argB*2, *bgA*o was used as recipient in transformations with *lacZ* constructs. Strains Δ1 (Pérez-Esteban *et al.*, 1993) or Δ1/ΔCreA (this work) contain single copy integrations at the *argB* locus of pBSΔ1 or pBSΔ1/ΔCreA, respectively and are otherwise isogenic to the recipient strain. In addition, all strains carry the *veA*1 allele. Conidiospores were obtained and growth tests were performed in, respectively, complete and minimal solid media of Cove (1966).

Penicillin production broth (PPB) is described in Espeso and Peñalva (1992); carbon sources were added separately, at the concentrations indicated (acetate as sodium salt). Inclusion of 100 mM Na_2HPO_4 in PPB results in pH 8.0 (without further additions). In control cultures, Na_2SO_4 or NaCl were included (see relevant sections) to evaluate the effect of extra Na^+ (200 mM). PPB was adjusted at acid pH values with 2-(*N*-morpholino)ethanesulfonic acid (MES). Addition of this compound at 100 mM or 200 mM results in pH 5.2 and 5.0, respectively.

For transfer experiments, incorporation of 100 mM MES and 100 mM NaH_2PO_4 in PPB results in pH 5.0. The pHs of the different media were adjusted with NaOH. At the highest pH tested (7.6), extra Na^+ from alkali addition was a further 172 mmol/l (i.e. total extra sodium ions were 272 mmol/l). Control experiments were carried out in cultures grown in sucrose−PPB containing 300 mmol/l of extra Na^+ ions (as Na_2SO_4 or NaCl) and in sucrose−PPB containing 100 mM NaH_2PO_4 + 200 mM NaCl (300 mmol/l of extra Na^+ ions, 100 mmol/l of extra phosphate ions) to eliminate the possibility that buffering (or associated) ions could effect changes on *ipnA* transcript levels.

Cultures in PPB were inoculated with conidiospores. Mycelia were harvested after filtration through Miracloth. The filtrate was used in pH determinations and to measure penicillin as described by Espeso and Peñalva (1992).

Molecular biology techniques

A plasmid overexpressing a GST::CreA(35−240) fusion polypeptide, containing the CreA DNA binding domain fused to the C-terminal portion of *Schistosoma japonicus* GST is described in Kulmburg *et al.* (1993) and was obtained from B.Felenbok (U.de Paris, Orsay). The plasmid was introduced into *Escherichia coli* DH5α′. This host was used to overexpress the polypeptide, which was purified by glutathione affinity chromatography (Smith and Johnson, 1988) as described by Kulmburg *et al.* (1992). Essentially homogeneous polypeptide was obtained, as judged by staining of SDS−polyacrylamide gels.

Binding of GST::CreA(35−240)-purified protein (or 'GST in controls) to terminally labelled *A.nidulans* DNA fragments was done as in Pérez-Esteban *et al.* (1993), in the presence of 3000-fold excess of poly(dI-dC)−poly(dI-dC) (Pharmacia). Detailed conditions of DNase I footprinting, performed essentially as in Ausubel *et al.* (1991), can be found in legend to Figure 1.

pBSΔ1/ΔCreA was constructed as outlined in Figure 2. PCR amplifications to produce fragments whose ligation reconstructed the *ipnA* promoter lacking sequences from 629 to 657 (from a *Xho*I site located at −2005 from the *ipnA* start codon) were carried out with the following primers (see Figure 2): 1, CGCCAAGCTCGGAATTAACCCT, corresponds to pBS-SK$^+$ sequences, *lacZ*′ coding strand, 5′-end at +14 from the ATG; 2, A-CATCATACACTATTACACCCTCAA; 3, GATCGACGCAGCAACA-GTATGTGA; 4, TGAGACTGATGAAGAAGCGGTGGTAACGG.

Plasmid pBSΔ1, containing the *ipnA* promoter region fused (translationally) to *'lacZ*, *A.nidulans* transformation, identification of single copy integration events by Southern analysis and conditions for β-galactosidase assays have been described (Pérez-Esteban *et al.*, 1993). RNA isolation and Northern analysis with actin- and *ipnA*-specific probes were performed essentially as in Espeso and Peñalva (1992).

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