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The *Aspergillus* PacC zinc finger transcription factor mediates regulation of both acid- and alkaline-expressed genes by ambient pH

Joan Tilburn, Sovan Sarkar, David A.Widdick[1], Eduardo A.Espeso[2], Margarita Orejas[2], Jeevan Mungroo[3], Miguel A.Peñalva[2] and Herbert N.Arst,Jr[4]

Department of Infectious Diseases and Bacteriology, Royal Postgraduate Medical School, Ducane Road, London W12 0NN, UK and [2]Centro de Investigaciones Biológicas del CSIC, Velázquez 144, Madrid 28006, Spain
[1]Present address: AFRC IPSR Nitrogen Fixation Laboratory, University of Sussex, Brighton BN1 9RQ, UK
[3]Present address: School of Biological Sciences, Queen Mary and Westfield College, London E1 4NS, UK

[4]Corresponding author

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The pH regulation of gene expression in *Aspergillus nidulans* is mediated by *pacC*, whose 678 residue-derived protein contains three putative Cys_2His_2 zinc fingers. Ten $pacC^c$ mutations mimicking growth at alkaline pH remove between 100 and 214 C-terminal residues, including a highly acidic region containing an acidic glutamine repeat. Nine $pacC^{+/-}$ mutations mimicking acidic growth conditions remove between 299 and 505 C-terminal residues. Deletion of the entire *pacC* coding region mimics acidity but leads additionally to poor growth and conidiation. A PacC fusion protein binds DNA with the core consensus GCCARG. At alkaline ambient pH, PacC activates transcription of alkaline-expressed genes (including *pacC* itself) and represses transcription of acid-expressed genes. $pacC^c$ mutations obviate the need for pH signal transduction.

Key words: *Aspergillus nidulans*/filamentous fungus/pH regulation/transcription factor/zinc finger

Introduction

Many microbes encounter large variations in ambient pH in their natural environments. Microorganisms capable of growing over a wide pH range require (i) a versatile, efficient pH homeostatic mechanism protecting intracellular processes against extremes of pH and (ii) a means of ensuring that activities undertaken beyond the boundaries of pH homeostasis are only attempted at appropriate ambient pH.

The ascomycete fungus *Aspergillus nidulans* is a pertinent model in that it is able to grow over a range of approximately eight pH units (Caddick *et al.*, 1986; Dijkema *et al.*, 1986; Rossi and Arst, 1990). Although its pH homeostatic mechanism has not been investigated in any detail, the formal genetics and physiology of its regulatory system for controlling syntheses of secreted enzymes, permeases and exported metabolites in response to ambient pH have been described (Caddick *et al.*, 1986; Shah *et al.*, 1991; Espeso *et al.*, 1993; Arst *et al.*, 1994). This system enables, *inter alia*, the secretion of alkaline phosphatase in alkaline environments and acid phosphatase in acidic environments. It is also responsible for a considerable elevation in penicillin biosynthesis at alkaline pH.

There are seven known genes where mutations can mimic the effects of growth at a pH other than the actual ambient pH (Caddick *et al.*, 1986; Shah *et al.*, 1991; Espeso *et al.*, 1993; Arst *et al.*, 1994). Mutations in any of the six genes *palA*, *B*, *C*, *F*, *H* or *I* mimic the effects of growth at acidic pH and result, for example, in elevated levels of acid phosphatase, reduced levels of alkaline phosphatase and lack of penicillin production. In contrast, mutations (now designated $pacC^c$) in *pacC* mimic the effects of growth at alkaline pH and lead, for example, to elevated levels of alkaline phosphatase, reduced levels of acid phosphatase (which formed the basis for selection of the first mutation) and penicillin overproduction. Epistasis relationships between *pal* and $pacC^c$ mutations and the diversity of *pacC* mutant phenotypes strongly suggested that the *pacC* product directly mediates regulation by ambient pH, whereas the six *pal* gene products participate in a pH signal transduction pathway (Caddick *et al.*, 1986; Shah *et al.*, 1991; Espeso *et al.*, 1993; Arst *et al.*, 1994).

Here we present a molecular analysis showing that PacC is a sequence-specific DNA binding protein and that $pacC^c$ mutations remove an acidic C-terminal segment which modulates its activity. In response to alkaline ambient pH, PacC activates the expression of genes whose products are synthesized preferentially at alkaline pH and represses the synthesis of gene products appropriate to acidic growth conditions. We believe that the model presented here could provide a conceptual framework and methodology for investigating pH regulation in a variety of other organisms.

Results and discussion

pH regulation affects transcript levels

Espeso *et al.* (1993) showed, using Northern blots, that ambient pH and mutations affecting pH regulation markedly affect transcript levels for the *ipnA* gene encoding isopenicillin N synthetase, a gene expressed predominantly at alkaline pH. Jarai and Buxton (1994) have similarly demonstrated the pH regulation of transcript levels for two genes encoding acid proteases in *Aspergillus niger*. Northern blots in Figure 1A and B show that levels of transcripts encoding an alkaline protease and an acid phosphatase also vary markedly with ambient pH and in pH regulatory mutants. As expected, alkaline growth pH or a $pacC^c$ mutation which mimics alkaline growth pH elicits high levels of alkaline protease transcript, whereas acid growth pH or a *pal* mutation which mimics acid

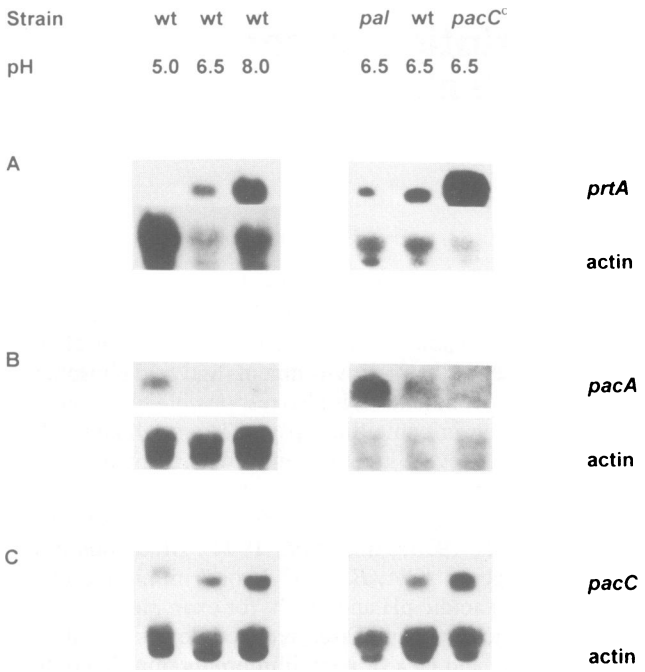


Fig. 1. Some effects of growth pH and mutations altering responses to growth pH on transcript levels. ~5 μg glyoxal-treated total RNA from a wild-type strain grown in media at the pH values indicated (on the left) or *pal*$^-$ (mimicking acidic growth conditions), wild-type and *pacC*^{c} (mimicking alkaline growth conditions) strains grown at pH 6.5 (on the right) were electrophoresed and analysed by Northern blotting. (**A**) Putative alkaline protease gene (*prtA*) probe; (**B**) putative acid phosphatase gene (*pacA*) probe; (**C**) *pacC* probe (nucleotides 488–3081; see Figure 2). Membranes were stripped and reprobed with the actin gene which shows two transcript sizes (Fidel *et al.*, 1988). Genotypes of strains used. (A and C) Lanes 1–3, *biA*1 (wild-type); lanes 4–6, *pabaA*1*palF*15, *pabaA*1 (wild-type), *pabaA*1*pacC*^{c}11. [For both the *pacC* and actin messages the RNA in the first lane of (C) appears to have run more slowly.] (B) *biA*l (wild-type), *pabaA*1 *yA*2 *wA*3 *palA*1; *biA*l *pacC*^{c}5. (Because of nuclease derepression, it is difficult to obtain good RNA preparations from phosphate-starved mycelia.)

growth pH reduces alkaline protease transcript levels. The opposite regulatory pattern is seen for acid phosphatase levels, and it can be concluded that both ambient pH and the *pacC*-mediated pH regulatory system affect transcript levels for both acid-expressed and alkaline-expressed genes.

Cloning and analysis of the pacC gene

pacC^c mutations which mimic the effects of growth at alkaline pH are detectable visually as they reduce conidiation on media below pH 7 (Caddick *et al.*, 1986). Therefore arginine-independent, morphologically normal transformants were selected using an *argB*2 *pacC*^{c}14 recipient and an *A.nidulans* library constructed in an *argB*$^+$-containing plasmid. Southern blotting (results not shown) showed three such transformants to contain single transforming plasmids integrated outside the *argB* region of the recipient genome. Plasmids able to complement both *argB*2 and *pacC*^{c}14 were recovered after transformation of *Escherichia coli* with undigested DNA from two of the *A.nidulans* transformants. Hybridization of Southern blots with one of these rescued plasmids (results not shown) indicated homologous (but not at *argB*) integration of transforming sequences in the three original single copy transformants. In each case, *argB*$^+$-transforming sequences were located by parasexual analysis (McCully and Forbes, 1965) to chromosome VI (which contains *pacC*). Meiotic analysis (results not shown) indicated tight linkage of the *argB*$^+$-transforming sequences to *glrA*1 which is closely linked (1 cM) to *pacC* (Bailey *et al.*, 1979). This is strong evidence that the transforming sequences contain *pacC* itself rather than an extragenic suppressor. Definitive evidence that the cloned region contains *pacC* is provided by a number of mutant sequence changes (*vide infra*). *pacC*$^+$-transforming activity was localized to a 2.6 kb *Hin*dIII–*Bam*HI fragment which hybridizes to a single mRNA of ~2.4 kb.

Figure 2A shows the sequence of a 3371 bp genomic region encompassing the *pacC*$^+$-transforming fragment. Sequencing of cDNA clones showed the presence of two introns, 85 and 53 nucleotides in length. The derived protein sequence of this region contains 678 residues. Towards the N-terminus are three putative zinc fingers of the Cys_2His_2 class (designated PacC-1 to -3 in Figure 2B). All three conform to the $CX_{2-4}CX_{12}HX_{3-5}H$ consensus (Jacobs, 1992) and contain a conserved hydrophobic residue (Klug and Rhodes, 1987) at position 4 of the putative α-helix. In addition, PacC-1 and -3 have leucine and phenylalanine, respectively, in position 3 of the putative second β-strand, a frequent, but not essential, feature of zinc fingers of this class (Klug and Rhodes, 1987; Suzuki *et al.*, 1994). PacC-1 has a rather unusual primary structure, perhaps similar to that of the first finger of GLI (Pavletich and Pabo, 1993). Particularly notable is the conservation of tryptophan residues in the first knuckles of both PacC-1 and PacC-2. Hydrophobic interaction between corresponding tryptophan residues in GLI, in conjunction with hydrogen bonding, stabilizes the interaction between fingers 1 and 2 (Pavletich and Pabo, 1993). PacC-1 resembles the first finger of *tra-1* (Zarkower and Hodgkin, 1992) in having an unusually long linker (a feature shared to a lesser extent by GLI-1). The seven residue PacC-2 linker is more typical and includes lysine and proline at positions t5 and t6, respectively, of Jacobs (1992). The putative recognition helix of PacC-2 is rather similar in sequence to that of Zif268-2, with lysine, aspartate and histidine residues at critical positions −1, 2 and 3, respectively (Pavletich and Pabo, 1991). PacC-3 is rather canonical. Database searching reveals a number of zinc finger structures with very similar putative recognition helices. Arginine at critical position −1 is present in all Zif268 fingers (Pavletich and Pabo, 1991) and Tramtrack finger 2 (Fairall *et al.*, 1993). An aspartate at position 3 is shared with GLI-4 as is lysine at 6 with GLI-4 and -5 (Pavletich and Pabo, 1993). A proline as the first residue of the recognition helix is not unusual (cf. GLI-5; Pavletich and Pabo, 1993).

The zinc finger region shows considerable sequence similarity to RIM1 (Su and Mitchell, 1993), a positive-acting regulator of meiosis in *Saccharomyces cerevisiae* (Figure 2B). In ungapped alignment over 97 residues, there are 55 identities and a further eight conservative differences. All potential chelating residues are conserved and the C-terminal portions of each finger and linker (including those of the unusual N-terminal fingers) are largely conserved. Therefore, PacC and RIM1 probably recognize very similar DNA sequences. Su and Mitchell

Table I. Mutant sequence changes in *pacC* and resulting proteins

Genotype	Phenotype	Mutation	Mutant protein
*pacC*504	c/+	G835C	M5I
(*pacC*5)		(C2528T)	(1 → 523*)
*pacC*7604	+/−	ΔC1426	1 → 173 QSLDLATQI*
*pacC*20001	+/−	C1604T	1 → 215*
*pacC*20203	+/−	T1609A	1 → 216*
*pacC*20102	+/−	1611+C	1 → 218 PGPSRSPAIEPVLRQRLLRSEYRPRASPSVV*
*pacC*20204	+/−	C1613T	1 → 218*
*pacC*20100	+/−	1613+TATTACC	1 → 218 LLPGPSRSPAIEPVLRQRLLRSEYRPRASPSVV*
*pacC*508	+/−	ΔC1631	1 → 224 SHRTRLTATSTTL*
*pacC*515	+/−	1641+A	1 → 227 KPVLRQRLLRSEYRPRASPSVV*
*pacC*7601	+/−	T2096G;ΔC2099	1 → 379 DALHTRLRHNFPRHMPQPRRLLVLLSQTHLR-TPLRLALRL*
*pacC*202	c	Δ2353 → 2555	1 → 464 IDRPGSPFRISGRG*
*pacC*14	c	C2437A	1 → 492*
*pacC*1400	+	A2436T	Y493L
(*pacC*14)			
*pacC*1401	+	A2436C	Y493S
(*pacC*14)			
*pacC*76	c	ΔG2442	1 → 494 EEHCSELGPRPVLPRSRWTCPATTRSLGSELPSRSPPV*
*pacC*5	c	C2528T	1 → 523*
*pacC*205	c	2528+T	1 → 523 LNSQADLRQFDRPGSPFRISGRG*
*pacC*204	c	ΔT2571	1 → 537 PIQDLRARMM*
*pacC*11	c	G2579T	1 → 540*
*pacC*1100	+	T2579G	1 → 678*
(*pacC*11)			
*pacC*1101	+	T2579C	G541R
(*pacC*11)			
*pacC*201	c	ΔA2603	1 → 548 LGLPRPQPRWRRGRTSSRSGLRRFG*
*pacC*203	c	ΔG2648	1 → 563 SSRSGLRRFG*
*pacC*200	c	2690+T	1 → 578 T*

Where the resulting mutant protein is dependent on an additional mutation in extant alleles that mutation is shown in brackets below the mutation under consideration. Nucleotide and amino acid numbering follows Figure 2A. c, constitutive phenotype, bypassing the requirement for the pH signal and mimicking alkaline growth conditions; +, wild-type phenotype; +/−, partial loss-of-function, mimicking acidic growth conditions; c/+, intermediate between c and + phenotypes. An asterisk denotes a stop codon.

(1993) showed that replacement of the second chelating cysteine of any of the three fingers of RIM1 by serine abolished function, consistent with the description of these sequences as zinc fingers. We have been unable to find any convincing sequence similarity between the PacC protein and other entries in the databases outside the zinc finger region.

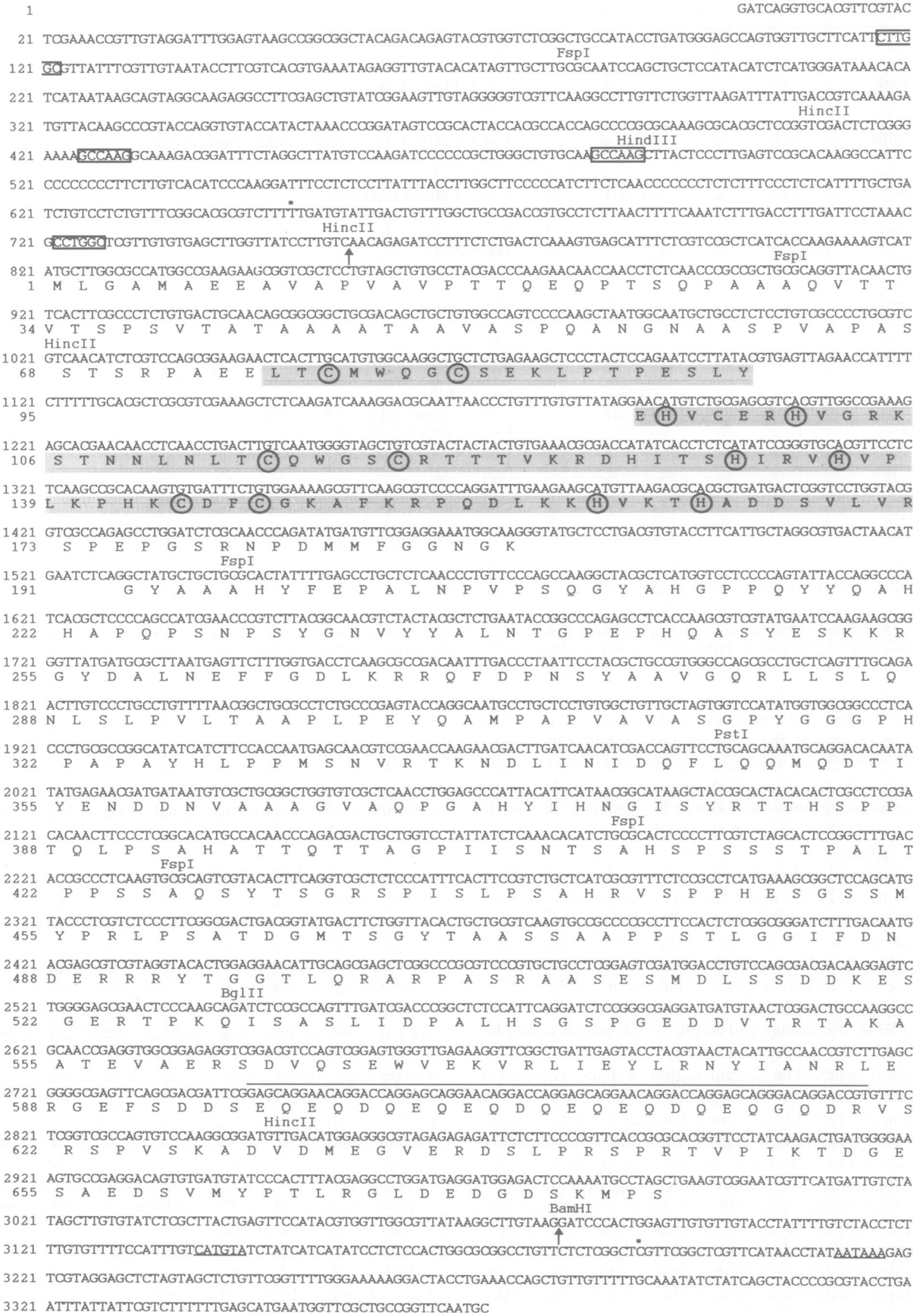
In common with other transcription factors, PacC is rich in the S/TPXX motif (Suzuki, 1989), containing 11 SPXX and four TPXX motifs. The N-terminus is very alanine-rich, with 22 alanines in the first 73 residues, and is predicted to have at least some α-helical structure. Alanine-rich helical regions have been implicated in transcriptional repression (Han and Manley, 1993; Licht *et al.*, 1994; Tzamarias and Struhl, 1994) which would be consistent with the repressing function by PacC for acid-expressed genes. The central region of PacC contains a tyrosine-rich, two proline−glycine-rich and, towards the C-terminus, two serine−threonine-rich regions (Figure 2C). The tyrosine-rich region coincides approximately with the more N-terminal proline−glycine-rich region. The C-terminus is very acidic. Beginning with nucleotide 2744 (and amino acid 596) are three perfect and one imperfect copies of an 18 bp direct repeat encoding a sequence composed almost exclusively of acidic and glutamine residues. Although regions containing acidic or glutamine residues have been implicated in transcriptional activation (reviewed by Tjian and Maniatis, 1994), mutational analysis (*vide infra*) indicates that the C-terminus is involved in the negative modulation of PacC activity rather than transcriptional activation. With the very acidic C-terminus the protein has a net charge of −19. The region between residues 251 and 270 might contain a bipartite nuclear localization signal (reviewed by Boulikas, 1994), SKKR ··· KRRQ.

Sequence analysis of mutations

Using classic genetics, two main categories of mutations in *pacC* have been obtained: $pacC^{c}$ mutations mimicking growth at alkaline pH and $pacC^{+/-}$ mutations mimicking growth at acidic pH. The $pacC^{c}$ phenotype includes reduced acid phosphatase, acid phosphodiesterase and γ-aminobutyrate (GABA) permease levels, enhanced sensitivity to aminoglycosides such as neomycin, elevated alkaline phosphatase and penicillin levels, and abnormal morphology with reduced conidiation at acidic pH (Caddick *et al.*, 1986; Shah *et al.*, 1991; Espeso *et al.*, 1993). $pacC^{+/-}$ mutations have phenotypes similar to, but less extreme than, those of *palA*, *B*, *C*, *F*, *H* and *I* mutations (Caddick *et al.*, 1986; Arst *et al.*, 1994) and mimic growth at acidic pH. Thus, $pacC^{+/-}$ mutations have phenotypes opposite to those of $pacC^{c}$ mutations, and whereas $pacC^{c}$ strains are morphologically abnormal at low pH, $pacC^{+/-}$ strains grow very poorly at alkaline pH.

Data in Table I show that truncation of the normal PacC sequence through frame-shift or chain termination mutation between residues 464 ($pacC^{c}202$) and 578 ($pacC^{c}200$) leads to a $pacC^{c}$ phenotype. [Reversion within

A


GATCAGGTGCACGTTCGTAC
FspI
HincII
HindIII
HincII
FspI
HincII
PstI
FspI
FspI
BglII
HincII
BamHI

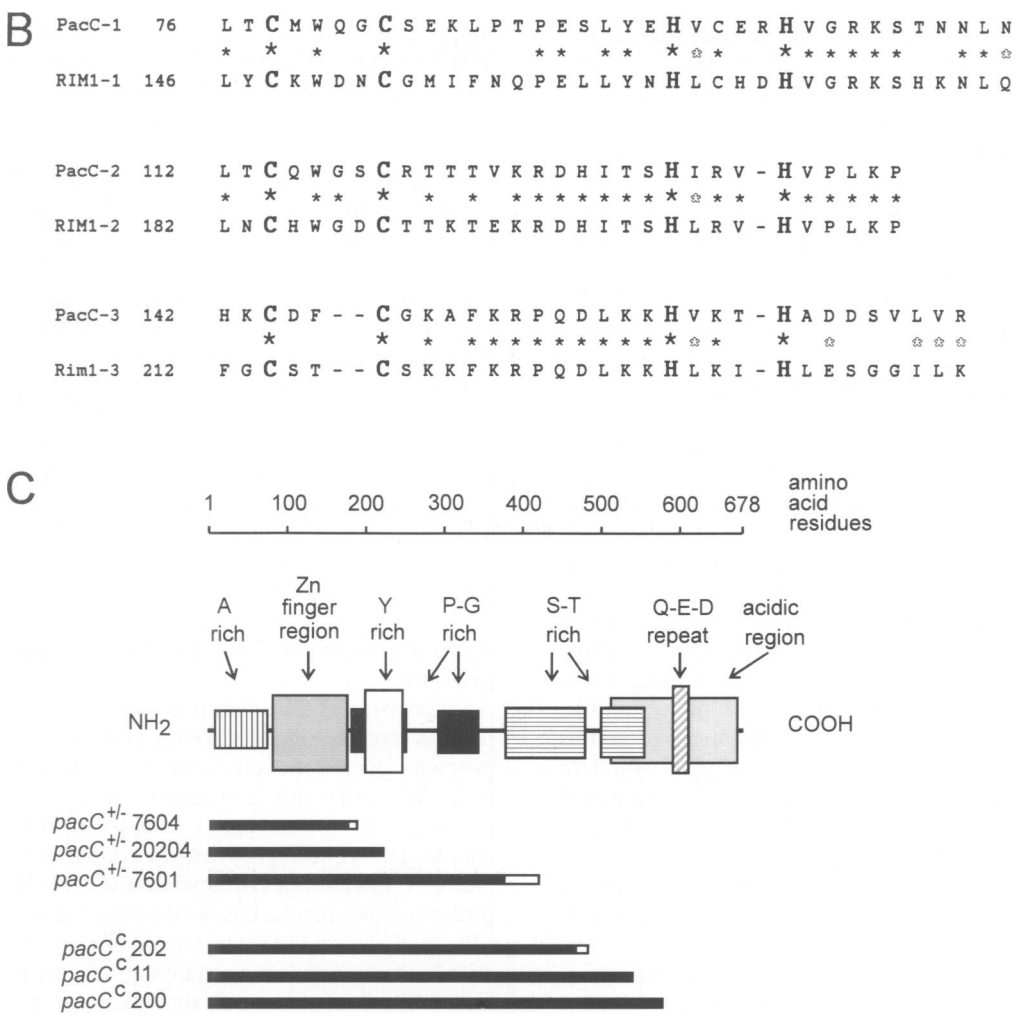


Fig. 2. Sequence of the *pacC* genomic region and some of its features. (**A**) Nucleotide and derived amino acid sequence of *pac*C. The zinc finger region is shaded and the likely zinc-chelating residues are circled. Possible PacC binding sites containing the GCCARG consensus (see Figure 5) in either orientation in the promoter are boxed. The four (three perfect plus one imperfect) tandem copies of the 18 bp repeat are overlined. Asterisks denote the limits of sequenced cDNA. Arrows indicate the limits of the deletion in the inactivation construct. Sites for restriction enzymes used in this work are shown. A sequence closely related to a hexanucleotide element required for mRNA 3′ end formation in yeast (Irniger and Braus, 1994) is underlined, as is an AATAAA eukaryotic consensus polyadenylation signal (reviewed by Wahle and Keller, 1992) which lies downstream of the two sequenced cDNA clones. Pyrimidine-rich regions typical of fungal gene promoters (Gurr *et al.*, 1987) occur between nucleotides 518 and 716. (**B**) Alignment of the PacC and *S.cerevisiae* RIM1 zinc finger regions. Solid asterisks indicate identities; open asterisks indicate similarities. Putative zinc-chelating residues are in bold. (**C**) Schematized PacC protein showing selected features. Only those features mentioned in the text are indicated. The portions of the protein remaining in certain mutants are indicated by solid bars, with open bar extensions denoting approximate lengths of abnormal sequence due to frameshift.

mutant stop codons at codons 493 (*pacC*^{c}14) and 541 (*pacC*^{c}11) restores a wild-type phenotype (Table I).] In contrast, truncation between residues 173 (*pacC*$^{+/-}$7604) and 379 (*pacC*$^{+/-}$7601) leads to a *pacC*$^{+/-}$ phenotype. Thus, *pacC*^{c} mutations eliminate the acidic/glutamine repeats as well as some or all of the remainder of the acidic region near the C-terminus. *pacC*$^{+/-}$ mutations additionally eliminate the two serine−threonine-rich regions; the more extreme also eliminate the C-terminal proline−glycine-rich region and part or all of the more N-terminal proline−glycine-rich region (coinciding with the tyrosine-rich region). Although none of the mutations affect the zinc finger region, *pacC*$^{+/-}$7604 would truncate the normal amino acid sequence only one residue after the end of sequence similarity to RIM1 (see Figure 2B).

The fact that *pacC*$^{+/-}$ mutations remove much more of PacC than *pacC*^{c} mutations supports the interpretation of *pacC*^{c} mutations as a gain-of-function class. The C-terminal region evidently modulates functionality of PacC in a negative fashion in response to ambient pH. Deletion of this region results in a protein which activates alkaline-expressed genes and represses acid-expressed genes in a pH-independent manner. As such deletions eliminate the acidic-glutamine repeats, as well as part or all of the remainder of the acidic region near the C-terminus (Table I and Figure 2C), these sequences are apparently not required for transcriptional activation (or repression) by PacC. *pacC*^{c} mutations can be considered a gain-of-function class in that the corresponding PacC^{c} proteins have gained the function contributed by the ambient pH signal.

The *pacC*504 (M5I) mutation requires separate consideration. It was selected by Caddick (1986) as attenuating, albeit partially, the alkalinity mimicking phenotype of

pacC^{c}5. Although it might indicate a role for the N-terminus in PacC activity, we speculate that it is more likely to reduce the translatability of *pacC* mRNA, 'leaky scanning' (Kozak, 1991) usually resulting in a significant frequency of translational initiation at codon 5 (to produce a protein of 674 residues). Comparisons of flanking nucleotides for initiation codons with those determined by tabulated consensus or as experimentally favourable in *Neurospora crassa* (Edelman and Staben, 1994) and *S.cerevisiae* (Cavener and Ray, 1991; Pinto *et al.*, 1992), suggest that codon 5 has a better context than codon 1.

The phenotype of a pacC null mutation

A null allele of *pacC* was constructed through homologous integration at the *pacC* locus of a linearized clone in which a region extending from 65 bp upstream of the putative initiator codon to 84 bp downstream of the TGA stop codon was replaced by the *pyr4* gene of *N.crassa*. A *pyrG*89 *pacC*^{c}14 strain was used as recipient so that the distinctive 'glassy' colony morphology would facilitate identification of integration events at the *pacC* locus amongst pyrimidine prototrophic transformants. Three independent transformants were very slow growing, forming very few conidiospores, irrespective of pyrimidine supplementation or growth pH (in contrast to *pacC*^{c} strains which resemble the wild-type at alkaline pH). Southern blotting and tight meiotic linkage of the morphological abnormality to *glrA*1 (results not shown) are consistent with a gene replacement event, in each case involving the *pacC* flanking sequences in the transforming fragment and the corresponding genomic sequences in the recipient. The phenotype of a null *pacC* allele, apart from slow growth and more extreme morphological abnormality, resembles that of *palA*, *B*, *C*, *F*, *H* and *I* mutations (Caddick *et al.*, 1986; Arst *et al.*, 1994) mimicking acidic growth pH. This is consistent with the interpretation of the extant acidity mimicking *pacC*$^{+/-}$ mutations, which remove up to 74.5% of the normal PacC sequence as partial loss-of-function mutations. The slow growth and nearly aconidial phenotypes might suggest an additional role for PacC or alternatively indicate that even in the absence of the *pal* signal pathway PacC retains slight residual activity.

pH regulation of pacC transcript levels

Figure 1C shows that *pacC* transcript levels are highest under alkaline growth conditions and lowest under acidic growth conditions. Consistent with this pattern, they are elevated relative to wild-type in a strain carrying a *pacC*^{c}11 mutation (which mimics alkalinity) and reduced in a *palF*15 (mimicking acidity) mutant. Thus *pacC* itself is an alkaline-expressed gene.

Epistasis of all types of pacC mutation to acidity mimicking pal mutations

It has been established previously that alkalinity mimicking *pacC*^{c} mutations are epistatic to mutations in the *palA*, *B*, *C*, *F*, *H* and *I* genes (Caddick *et al.*, 1986; Arst *et al.*, 1994). It was therefore of interest to determine whether *pacC*$^{+/-}$ mutations, which mimic acid growth conditions less extremely than *pal* mutations, are similarly epistatic, and whether a *pal* mutation can be detected in a *pacC* null background. *palB*7 was chosen for these epistasis/additivity tests because the existence of strains with closely linked markers on either side facilitates the identification of strains carrying it in the presence of a masking *pacC* mutation. (Mutations in the *palA*, *B*, *C*, *F* and *H* genes are in any case phenotypically indistinguishable.) Strains carrying *pacC*$^{+/-}$7604, *pacC*$^{+/-}$20102, *pacC*$^{+/-}$20100 or the *pacC* null allele were indistinguishable from their respective double mutants carrying *palB*7. *pacC*$^{+/-}$7604 was also shown to be epistatic to *palC*4. Because *palI* mutations allow some growth at pH 8 (Arst *et al.*, 1994), whereas the *pacC* null allele does not, an epistasy test is possible for the *pacC* null allele uniquely in this case. *palI*30 *pacC* null double mutants failed to grow at pH 8 and were indistinguishable from *pacC* null single mutants. Thus, in every instance tested, acidity mimicking *pal* mutations are hypostatic to *pacC* mutations. The fact that *pacC*^{c}, *pacC*$^{+/-}$ and *pacC* null mutations are all epistatic to *pal* mutations argues very strongly that the *pal* genes are not structural genes under *pacC* control and that their physiological roles are confined to involvement with PacC.

Sequence-specific DNA binding by a PacC fusion protein

As expression of the alkaline-expressed *ipnA* gene is under positive control by PacC (Espeso *et al.*, 1993), the *ipnA* promoter would be predicted to contain PacC binding sites. To verify this prediction and to confirm that the putative zinc finger region actually mediates sequence-specific DNA binding, we expressed in *E.coli* and purified a chimeric glutathione S-transferase (GST)::PacC(30–195*) protein containing the entire zinc finger region (Figure 3A). This fusion protein was assayed for gel mobility shifts with a collection of overlapping fragments covering a region 2 kb upstream of the initiation codon of *ipnA*. Two fragments formed specific retardation complexes, indicating that the region of PacC between residues 30 and 195 contains a sequence-specific DNA binding domain. These fragments are included in two regions of the *ipnA* promoter shown to contain functional positive regulatory sites by deletion analysis of reporter gene expression (Pérez-Esteban *et al.*, 1993).

Increasing amounts of GST::PacC(30–195*) result in the formation of up to three major retardation complexes with a 198 bp *Sac*I–*Spe*I fragment (−653 to −455 relative to the major *ipnA* transcription start point; Figure 3B). This suggests that the fragment contains three PacC binding sites with different affinities, with the high mobility complex (I) corresponding to the binding of the protein at one site and the other complexes resulting from the simultaneous occupancy of two (complex II) or all three (complex III) PacC binding sites. Similar experiments (results not shown) showed the presence of two major binding sites in a 284 bp *Hin*dIII–*Bss*HII fragment (−338 to −54 relative to the tsp). As a control, a 340 bp *Tth*111I–*Ava*I fragment of the *alcR* promoter, which contains a binding site for the two zinc finger repressor CreA, forms a retardation complex with a GST::CreA(35–240*) fusion (which contains the CreA DNA binding region; following Kulmburg *et al.*, 1993) but not with the GST::PacC(30–195*) fusion (Figure 3C). We conclude that at least five *in vitro* PacC binding sites are present in the *ipnA* promoter.

DNase I footprinting analysis (Figure 4) confirmed this

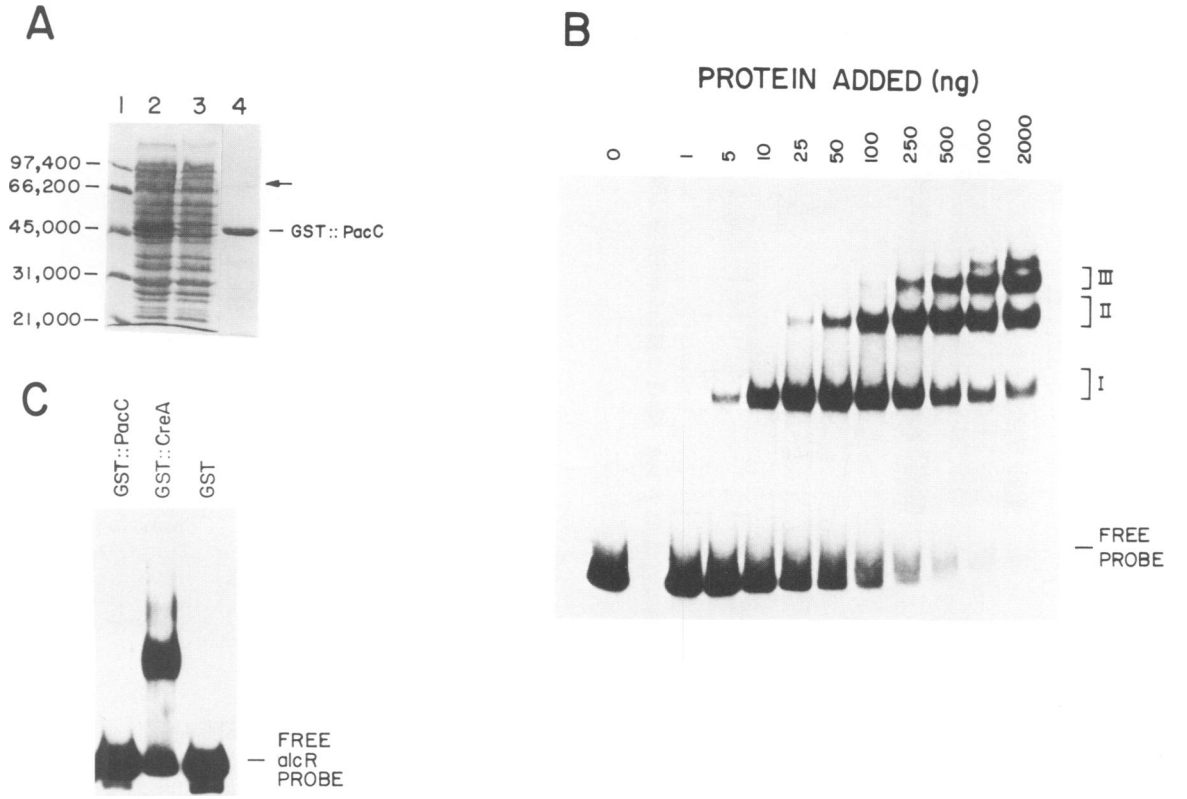


Fig. 3. A PacC fusion protein containing the zinc finger region binds DNA. (**A**) SDS−PAGE of purified GST::PacC(30–195*) made in *E.coli*. Lane 1, molecular weight standards; lanes 2 and 3, cleared lysate before and after passage through the glutathione affinity column; lane 4, material retained in the column after glutathione elution. The predicted molecular weight of the fusion protein is 45 203 g/mol. The faint low mobility band (indicated by an arrow) corresponds to *E.coli* GST. (**B**) Gel retardation assay using an *ipnA* promoter fragment. A ^{32}P-labelled 198 bp *Sac*I−*Spe*I fragment was incubated in the presence of the indicated amounts of purified GST::PacC(30–195*). The formation of three major complexes following autoradiography of the dried gel is apparent. A fourth minor band, possibly corresponding to the binding of four protein molecules to the fragment, is observed only at very high protein concentrations and is not detected in footprinting experiments (Figure 4). It must therefore reflect the presence of another very weak binding site(s) which is recognized only in the presence of a large excess of protein. No retardation complexes were observed with purified GST (data not shown). (**C**) Gel retardation assay using a 340 bp *Tth*111I−*Ava*I fragment of the *alcR* promoter containing a CreA binding site (Kulmburg *et al.*, 1993). Reactions were performed in the presence of 1 μg GST::PacC(30–195*) or GST, as indicated.

interpretation and located the positions of these sites; it also documented the differences in their relative PacC affinities. Figure 5 summarizes the data and derives a consensus binding site. The *Sac*I−*Spe*I fragment contains three protected regions (designated ipnA1–3). Site ipnA2 has a much higher affinity for the GST::PacC fusion protein than the other two, as shown by much stronger protection (Figure 4). The *Hin*dIII−*Bss*HII fragment contains a single protected window (Figure 4). However, the fact that the length of the protected DNA is roughly twice that found in sites ipnA1, ipnA2 and ipnA3, together with the formation of two, rather than one, retardation complexes with this fragment, strongly suggested that this window represents binding by two molecules of fusion protein to a pair of neighbouring sites. Sequence analysis (Figure 5) confirmed this interpretation, the two sites being designated ipnA4A and ipnA4B. The relative affinity of the double ipnA4 site for the fusion protein is similar to that of ipnA2 and greater than those of ipnA1 and ipnA3 (Figure 4). Comparison of the sequences protected by GST::PacC yielded the consensus 5′-GCCARG-3′ (Figure 5), strongly suggesting that this is the core sequence recognized by the PacC DNA binding region. Site ipnA4 contains a pair of converging consensus hexanucleotides separated by 9 bp. The presence of four such hexanucleotide sequences in the *pacC* promoter (Figure 2) should be particularly noted in view of the autogenous regulation of *pacC* (Figure 1C).

To confirm that this 6 bp consensus sequence represents at least part of the PacC binding site, we showed that a single base pair change within it drastically reduces PacC binding (Figure 6). A 31 bp double-stranded fragment containing the high affinity ipnA2 site (including the 19 bp protected region plus flanking base pairs) is bound by GST::PacC(30–195*) (Figure 6, lane 7), indicating that ipnA2 can be recognized by PacC in isolation (i.e. in the absence of ipnA1 and ipnA3). To choose a single base pair change in the consensus which was likely to prevent binding, we scanned the *ipnA* promoter for related sequences not bound by the fusion protein, noting two sequences differing from the consensus by a T rather than an A at position 4. Therefore, we constructed a 31 bp double-stranded fragment identical to that mentioned above but containing this A → T transversion. A cross-competition experiment (Figure 6) shows that this single base pair change substantially prevents an excess of double-stranded oligonucleotide competing with its ^{32}P-labelled ipnA2 wild-type sequence counterpart. Whereas the presence of a 10-fold excess of unlabelled ipnA2 wild-type results in 50% inhibition of labelled complex

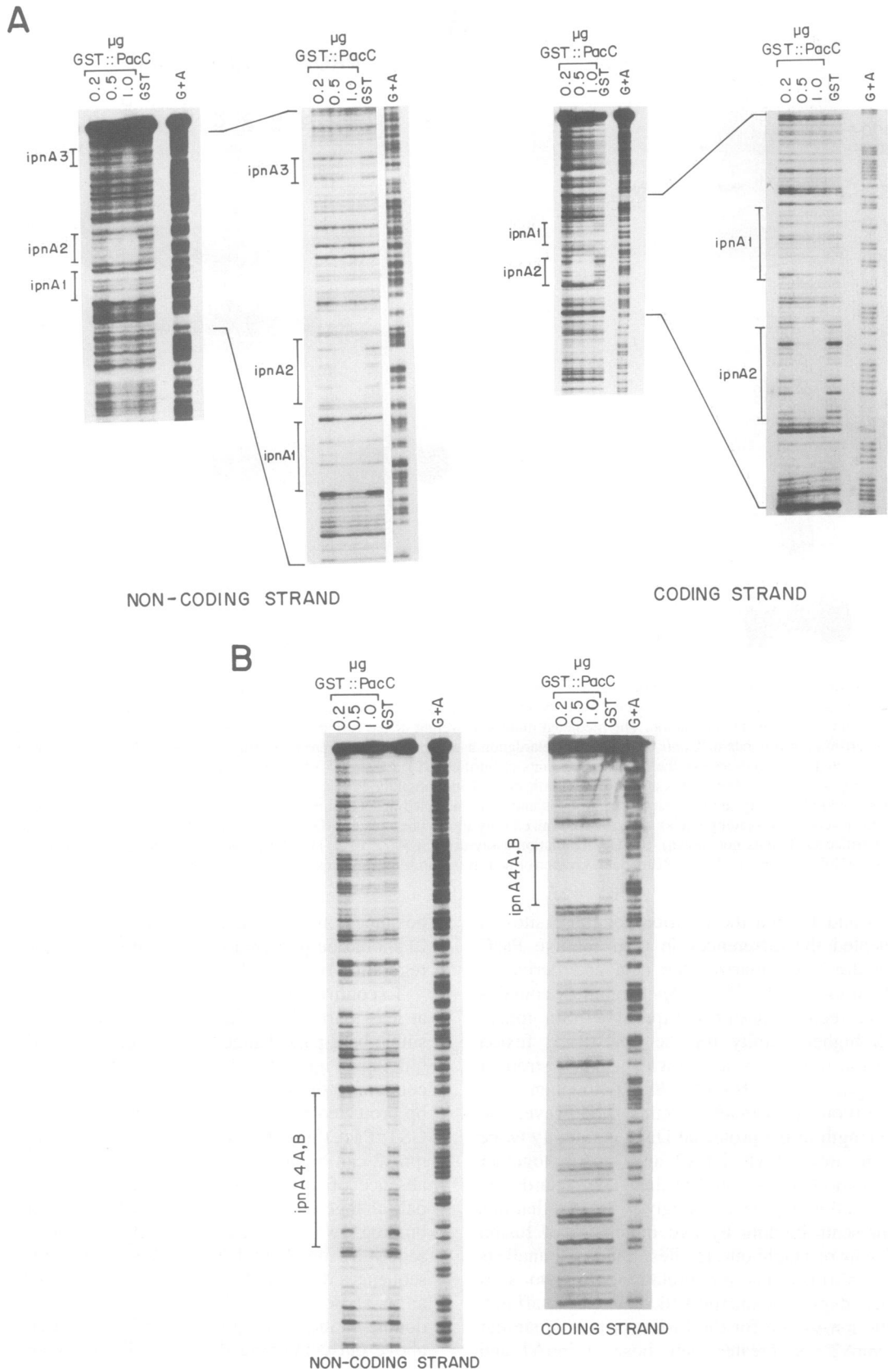


Fig. 4. DNase I footprinting analysis of PacC binding sites in the *ipnA* promoter. Representative footprints for sites in the 198 bp *Sac*I−*Spe*I fragment (**A**) and the 284 bp *Hin*dIII−*Bss*HII fragment (**B**) are shown. Coding and non-coding strands refer to the *ipnA* gene. Fragments were incubated in the presence of indicated amounts of GST::PacC(30–195*). Controls in the presence of 1 μg GST are included. A Maxam−Gilbert G + A reaction of the same fragment was run in parallel to identify nucleotide positions. The protected regions corresponding to each site are indicated.

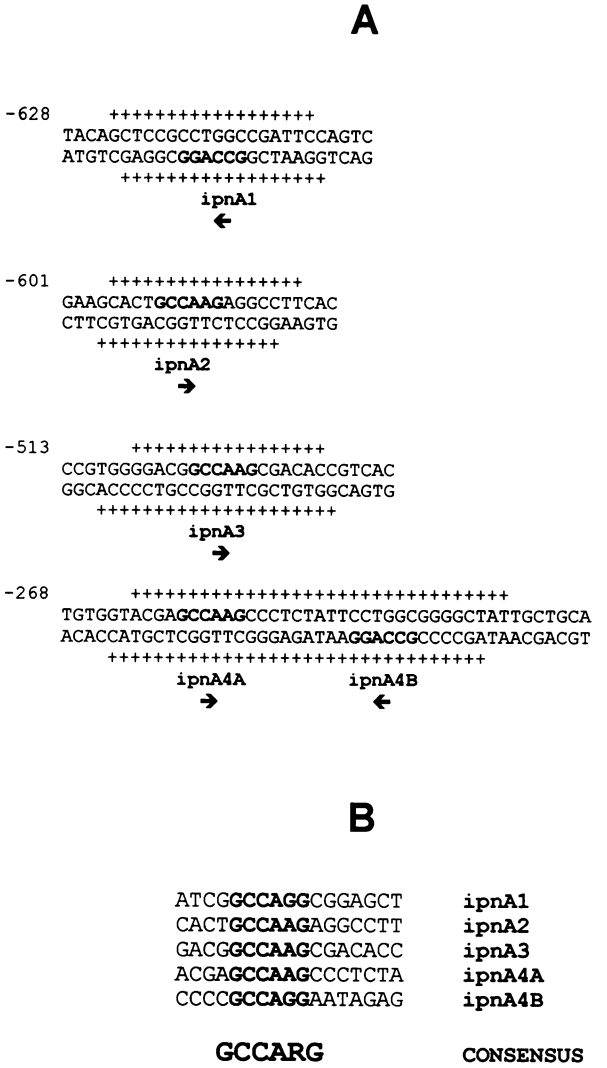


Fig. 5. PacC binding sites in the *ipnA* promoter. The sequences of the *in vitro* binding sites determined in Figure 4 are shown in detail. Numbers on the left indicate nucleotide positions relative to the *ipnA* major transcription start point. Positions protected from DNaseI digestion are indicated by + symbols. The hexanucleotide presumable core of the PacC binding sites is shown in bold. Also shown is an alignment of the relevant part of the five PacC binding sites and the derived consensus.

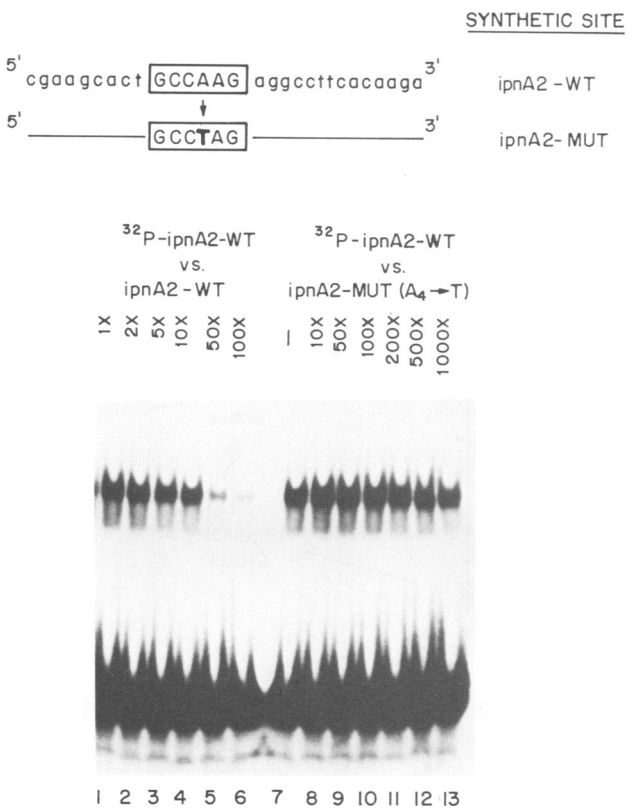


Fig. 6. A base change in the consensus hexanucleotide prevents PacC binding. 0.3 ng of synthetic 31mer double-stranded oligonucleotide containing site ipnA2 were labelled with ^{32}P and incubated with 10 ng (an excess) of GST::PacC(30–195*) in the absence (lane 7) or presence of the indicated excess of either the same unlabelled oligonucleotide (lanes 1–6) or an oligonucleotide differing only in that thymine replaced adenine in the fourth position of the PacC consensus ($A_4 \rightarrow T$) (lanes 8–13). All reactions contained in addition 3 μg of poly(dI−dC)·poly(dI−dC). In the absence of fusion protein, only the band corresponding to free probe was present (results not shown).

formation (lane 4), a similar effect could only be achieved by a 1000-fold excess of mutant site (lane 13). No binding of the fusion protein to the mutant ipnA2 site could be detected (data not shown). Therefore replacement of the A at position four of the consensus by T prevents PacC binding.

Although the hexanucleotide consensus represents an important component of the PacC binding site, it is probably not the whole site. ipnA2 and ipnA3 both contain it, yet differ 5- to 10-fold in relative affinity for the fusion protein. It is possible that a longer consensus will emerge when more (strong and weak) binding sites have been identified. If all three putative zinc fingers participate in base-specific contacts, there is scope for a longer site (see Pavletich and Pabo, 1993, and references therein).

A model for pH regulation

Figure 7 presents a model superseding that of Caddick *et al.* (1986) and Shah *et al.* (1991) and consistent with the available data on pH regulation in *A.nidulans*. PacC activates transcription of alkaline-expressed genes and represses transcription of acid-expressed genes in the presence of a signal mediated by the products of the *palA*, *B*, *C*, *F*, *H* and *I* genes in response to alkaline ambient pH (Arst *et al.*, 1994, and references therein). Thus at alkaline ambient pH, expression of alkaline genes (e.g. *palD*, *ipnA*, *prtA*) is elevated whereas that of acidic genes (e.g. *pacA*, *gabA*) is diminished. *pacC* is itself an alkaline-expressed gene, subject to autogenous transcriptional activation, amplifying the alkaline ambient pH signal. Under acidic conditions the level of PacC is diminished and it is in a non-functional form (perhaps owing to lack of pH signal transduction), leading to derepression of acid-expressed gene products and lack of activation of alkaline-expressed structural genes (including *pacC* itself). *pacC*^{c} mutations, by removing the C-terminus, obviate the need for the signal, leading to constitutivity of alkaline-expressed genes (including *pacC* itself) and super-repression of acid-expressed genes. *pacC*$^{+/-}$ mutations, by removing more of PacC, diminish its function. The alkaline growth impairment of *pacC*$^{+/-}$ strains and the lack of growth of *pacC* null and *pal* mutant strains under alkaline conditions, as well as the reduced conidiation and abnormal morphology of *pacC*^{c} strains under acidic conditions and

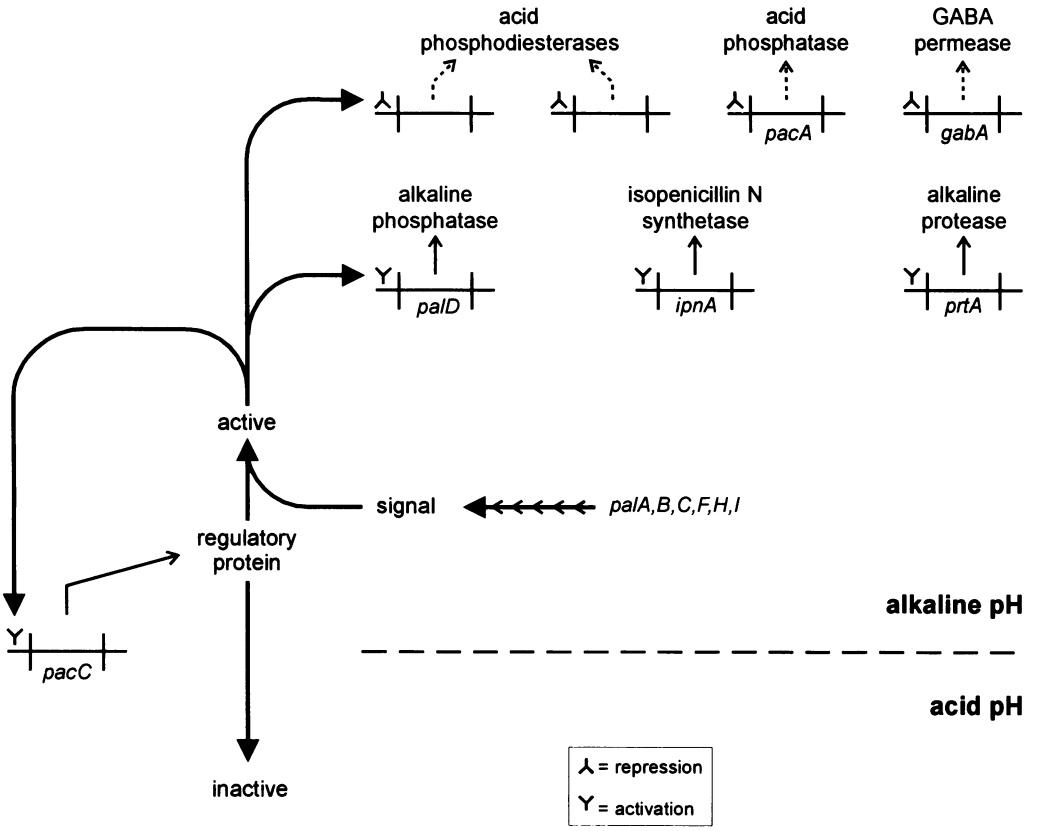


Fig. 7. A model for the regulation of gene expression by ambient pH in *A.nidulans*.

pacC null strains under all conditions, suggest that PacC might also play roles in pH homeostasis and development. Experiments to distinguish functional and inactive forms of PacC are in progress.

Materials and methods

A.nidulans strains, phenotype analysis and genetic techniques

A.nidulans strains carried previously described markers. Standard media, phenotype testing and genetic procedures were used (Caddick *et al.*, 1986; Clutterbuck, 1993; Arst *et al.*, 1994, and references therein). *pacC* mutations used in this work have been described previously (Dorn, 1965; Caddick *et al.*, 1986), with the exception of those listed in Table II. pH 9.0 medium was made by adding 50 mM (final concentration) 1,3-bis-[tris(hydroxymethyl)methylamino]-propane to minimal medium and adjusting to pH 9.0 with NaOH.

Transcript level analysis

Mycelia were grown from inocula of 2.5–5.0×10^6 conidiospores/ml in appropriately supplemented shaken liquid minimal medium and harvested as described by Cove (1966). For the monitoring of transcripts under different conditions of growth pH, additional buffering was provided by 10 mM citrate for pH 5.0, 10 mM 2-(*N*-morpholino)ethanesulfonic acid for pH 6.5 or 10 mM Tris–HCl for pH 8.0. pH values at harvesting were within 0.2 U of initial values. 1% (w/v) D-glucose served as the carbon source for monitoring *pacC* and putative *pacA* transcript levels; 0.1% (w/v) D-fructose served as the carbon source for monitoring *prtA* transcript levels. 100 μg/ml uric acid served as the nitrogen source. Growth times and temperatures for *pacC* transcripts were 16.5 h at 27°C (mutants and wild-type grown at pH 6.5) or 13 h at 30°C (wild-type compared at different pH values), 20 h at 27°C (*prtA*) and 12 h at 30°C followed by 5 h at 37°C in (otherwise identical) phosphate-free medium (*pacA*). RNA was extracted after grinding mycelia in liquid nitrogen using RNAzol B (Cinna/Biotecx) according to the manufacturer's instructions. Northern analysis was carried out with glyoxal-treated total RNA (Sambrook *et al.*, 1989) using Hybond N membranes (Amersham).

A.nidulans transformation

Tilburn *et al.* (1990) was followed except that after the second incubation with T-PEG the mixture was diluted with 5 ml STC and plated on 1 M sucrose medium (Tilburn *et al.*, 1983) in volumes not greater than 1 ml per 3 ml soft overlay containing 0.3–0.5% agar. Unless otherwise stated, the medium was buffered at pH 7.0.

For *pacC* cloning, protoplasts of a *biA*1 *pabaA*1 *argB*2 *pacC*^{c}14 *choA*1 strain were transformed with an *A.nidulans* library in pFB39, a pUC8-derived plasmid which contains the *A.nidulans argB*$^+$ (ornithine transcarbamylase) gene (Suárez *et al.*, 1991); *pacC*$^+$ transformants were detected visually amongst arginine-independent transformants.

pacC inactivation used protoplasts of a *yA*2 *pabaA*1 *pyrG*89 *argB*2 *pacC*^{c}14 strain. The transforming DNA, p4D (see below), in which the *pacC* coding region has been replaced by the *N.crassa pyr4* (orotidine 5′-monophosphate decarboxylase) gene, was digested with *Eco*RI and *Sph*I (cutting in the pUC19 polycloning sites either side of the insert sequences) and used sparingly (<250 ng). *pacC* null transformants were detected visually amongst pyrimidine-independent transformants at pH 6.5.

Gene libraries for other pacC clones and cloning A.nidulans-repressible acid phosphomonoesterase (pacA) and alkaline protease (prtA) genes

An *A.nidulans* library from a *pabaA*1 *areB*401 strain, constructed by Johnson (1988) by cloning *Sau*3a partially digested, size-selected (16–22 kb) fragments in the *Bam*HI site of λ EMBL4, was used for cloning the putative *pacA* and *prtA* genes and for obtaining non-rescued (see below) *pacC* clones. The *pacA* clone was obtained by hybridization with a fragment from the corresponding *A.niger* gene (MacRae *et al.*, 1988). A subcloned fragment transforms an *A.nidulans pacA*$^-$ strain restoring acid phosphatase activity; the derived amino acid sequence of a portion of this fragment shows identities with the corresponding *Aspergillus ficuum* (Ullah *et al.*, 1994) and *A.niger* sequences (S.Sarkar, J.Tilburn and H.N.Arst,Jr, unpublished results). The *prtA* gene was obtained by hybridization with the insert of pID13 containing part of the coding region of the *Aspergillus fumigatus* alkaline protease gene (Tang *et al.*, 1992). The derived PrtA sequence (M.D.Carmen Ovejero, E.Bignell, J.Tilburn and H.N.Arst,Jr, unpublished results) is very similar

Table II. New *pacC* mutations used in this work

Mutation	Parental strain genotype	Mutagen	Selection method	Reference
pacC^{c}76	*pabaA1 yA2 wA3 palA1*	UV	growth at pH 8	this work
pacC^{c}200, 201	*yA2 gatA2 pantoB100*	UV	resistant to 10 mM GABA	this work
pacC^{c}202, 203	*pabaA1 sasA60*	spontaneous	resistant to 10 mM GABA	C.R.Bailey and H.N.Arst,Jr, unpublished
pacC^{c}204, 205	*pabaA1 gatA2 chaA1 palB7*	UV	growth at pH 9	this work
pacC504	*pabaA1 pacC*^{c}*5*	4-nitroquinoline-1-oxide	growth on 50 mM GABA as C and N	Caddick (1986)
pacC$^{+/-}$508, 515	*pabaA1 pacC*^{c}*5*	UV	resistant to 200 μg/ml neomycin + 0.04% sodium deoxycholate	Caddick (1986)
pacC1100	*pabaA1 pacC*^{c}*11 pantoB100*	UV	resistant to 2 mg/ml neomycin	this work
pacC1101	*biA1 lysA2 pacC*^{c}*11*	spontaneous	reversion in selfed cleistothecium	this work
*pacC*1400, 1401	*yA2 choA1 pacC*^{c}*14*	UV	resistant to 2 mg/ml neomycin	this work
pacC$^{+/-}$7601, 7604	*inoB2 pacC*^{c}*76*	UV	resistant to 2 mg/ml neomycin	this work
pacC$^{+/-}$20001	*yA2 pacC*^{c}*200 gatA2 pantoB100*	UV	resistant to 1 mg/ml neomycin	this work
pacC$^{+/-}$20100, 20102	*yA2 pacC*^{c}*201 gatA2 pantoB100*	UV	resistant to 1 mg/ml neomycin	this work
pacC$^{+/-}$20203, 20204	*pabaA1 pacC*^{c}*202*	UV	resistant to 1 mg/ml neomycin	this work

to its counterparts in other fungi (Frederick *et al.*, 1993), and the *prtA* genomic sequence is consistent with it being the same gene as that described by M.E.Katz, R.N.Rice and B.F.Cheetham (EMBL accession no. L31778).

pacC cDNA clones pFA2 and pJB2 were derived from clones obtained from a λ gt10 library (Osmani *et al.*, 1988) isolated by hybridization to the insert of p5a (see below).

Plasmids

pT53c and pT71a were rescued (following Johnstone *et al.*, 1985) by transformation of *E.coli* DH5α with undigested DNA from *A.nidulans* primary *pacC*$^{+}$ *argB*$^{+}$ transformants. Both contain *argB*, pUC8 and the entire predicted *pacC* coding region. pT53c contains ~4.7 kb from the *pacC* region (starting ~1.8 kb upstream of the initiator codon) and pT71a contains ~6.1 kb from the *pacC* region (extending ~3.9 kb downstream of the termination codon).

p3ii was obtained by subcloning into pUC19 an *Eco*RI (λ site)−*Sma*I fragment from a *pacC* λ EMBL4 clone (3ii) and extends ~5.1 kb from nucleotide 1. Plasmids derived from p3ii include p4 (~3.8 kb insert from nucleotide 1) and p5 (nucleotides 1–3079 in *Bam*HI site). A series of ordered deletions was derived from p5 by the deletion of suitably positioned restriction fragments and by unidirectional digestion by the method of Henikoff (1987).

The *pacC* inactivation construct p4D was made by transferring from p5 a *Hin*dIII−*Sal*I fragment (nucleotide 488 to the pUC19 *Sal*I cloning site) to pUC19 digested with *Hin*dIII and *Sal*I, such that the pUC19 cloning sites *Bam*HI and *Xba*I were inverted and duplicated about the *Sal*I site. The resulting plasmid p5a was then digested with *Hin*cII which cleaves after nucleotide 755 and at the pUC19 site, removing nucleotides 756–3079 from the *pacC* gene. The insert was then transferred as a *Hin*dIII−*Bam*HI fragment to p4 where it replaced nucleotides 488–3079 to give p4Δ. An ~3.2 kb *Bgl*II fragment from pFB6 containing the *N.crassa pyr4* gene (Buxton and Radford, 1983) was inserted into the *Bam*HI site of p4Δ to give p4D in which *pyr4* is flanked by ~750 bp of upstream and ~700 bp of downstream *pacC* genomic sequences.

Sequence analysis

The *pacC* genomic sequence was obtained by double-strand sequencing of plasmids (pT53c, pT71a and 3ii-derived) and PCR amplification products, such that the region was sequenced on both strands using synthetic oligonucleotide primers and the Sequenase system (USB). No part was sequenced entirely from rescued plasmid (pT53c or pT71a) or λ (3ii)-derived clones. Mutant sequence changes were determined by direct sequencing of PCR products (Kudla *et al.*, 1990). Templates for PCR amplification were naked DNA prepared following Raeder and Broda (1985), conidial suspensions (Aufauvre-Brown *et al.*, 1993) or Chelex-treated conidial suspensions [essentially by the method of Walsh *et al.* (1991) using 10^5 conidiospores in 10 μl 0.01% Tween-80 with 200 μl 5% Chelex 100].

The *pacC* coding region was predicted from sequenced cDNA clones: pJB2 contains nucleotides 869–3170, spanning excised introns 1 (nucleotides 1103–1187) and 2 (nucleotides 1477–1529), and lacks a poly(A) tail. pFA2 contains nucleotides 1222–3189, spanning excised intron 2, and has a poly(A) tail of 10 residues. cDNA sequences extending further upstream were obtained by 5′ RACE modified after Frohman (1990). Wild-type (*pabaA1* grown at pH 6.5) total RNA was reverse transcribed using the primer 5′-AAGGATCCGTCGACATCGATAATACGACTCACTATAGGGA$(T)_{17}$-3′, modified after the (dT) 17-adapter 57 primer of Frohman and Martin (1989) and Superscript RT/buffer (Gibco BRL). After 2 min at 37°C, the Superscript RT was added, followed by 10 min at 37°C and a gradient of 1°C/2 min to 50°C where incubation was continued for 40 min (following T.Langdon and H.N.Arst,Jr, unpublished results). After purification with a Microcon 30 spin column (Amicon) the cDNA was polydeoxycytidylated using terminal transferase/buffer (Stratagene). 10 μl of the tailed cDNA/RNA mixture were treated with RNase H (Gibco BRL) in 50 μl first-strand buffer (Gibco BRL) for 30 min at 37°C. The cDNA was amplified by PCR using 5′ adapter primer G12 [5′-GGTCGGTACCAAGCTT$(G)_{12}$-3′ supplied by T.Langdon] and 3′ gene-specific primer 4R3FF (5′-GTCTTAACAYGCTTCTTCAAATCCTG-3′; nucleotides 1392–1367). The reaction was 'hot-started' after 5 min at 95°C by adding *Taq* polymerase during a 1 min incubation at 80°C, followed by 60°C for 5 min, 72°C for 5 min and 29 cycles of 94°C for 40 s, 60°C for 1 min and 72°C for 2 min (followed by a final extension cycle of 72°C for 5 min). The longest (~650 bp) band was reamplified and digested with *Hin*dIII (cutting in the 5′ adapter primer) and *Fsp*I (cutting after nucleotide 906). The ~200–250 bp *Hin*dIII−*Fsp*I fragments were subcloned into pUC19 and sequenced. The longest sequenced fragment extended to nucleotide 660 and had 13 'G's at its 5′ end, consistent with its being a cDNA amplification product. Intron excision was confirmed in the original ~650 bp band by sequencing its ~440 bp *Fsp*I−4R3FF fragments.

Preparation of a PacC fusion protein and its use in gel mobility shift and footprinting experiments

An *Fsp*I fragment encoding amino acids 30–195 of PacC was inserted into the *Sma*I site of pGEX-2T (Pharmacia) in the correct orientation to give plasmid pGEX−PacC. The resulting chimeric fusion protein contains the C-terminal sequence GIHRD following residue 195 of PacC. pGEX−PacC was transformed into *E.coli* JM103 and the expression and purification of the GST::PacC polypeptide was carried out essentially as suggested by Pharmacia, but including 0.5 M (final concentration) NaCl in the glutathione affinity column. Protein concentrations were estimated by the Bradford (1976) method using lysozyme as standard.

Fragments and synthetic double-stranded oligonucleotides were labelled by end filling with Klenow polymerase (Boehringer) in the presence of a mixture of dNTPs containing [α-^{32}P]dCTP. Binding reactions were performed as described (Pérez-Esteban *et al.*, 1993; Espeso and Peñalva, 1994) using 1 ng of probe DNA and a 3000-fold excess of poly(dI−dC)·poly(dI−dC) (Pharmacia) as non-specific competitor. The amounts of GST::PacC used are indicated, 1 ng of

protein representing ~20 fmol. Gel retardation assays were performed in 5 (restriction fragments) or 8% (double-stranded oligonucleotides) polyacrylamide gels run in 0.5× TBE. DNase I footprinting analysis followed Espeso and Peñalva (1994).

Double-stranded oligonucleotides were prepared by annealing equimolar amounts of single-stranded oligonucleotides in (final concentrations) 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT and 1 mM spermidine. The mixture was heat-denatured at 85°C for 3 min, slowly cooled to 40°C and further cooled to 4°C. It was then run on a polyacrylamide gel and double-stranded oligonucleotides were eluted by the method of Maxam and Gilbert (1980); their concentrations were estimated from OD at 260 nm.

The synthetic 31mer ipnA2 site (Figure 6) has the following (one-strand) sequence: 5′-CGAAGCACTGCCAAGAGGCCTTCACAA-GACG-3′ in which the last two nucleotides at both ends were left protruding for terminal labelling (and the CG at the 3′ end is non-genomic). The bold A is a T in the mutant synthetic site ipnA2 ($A_4 \rightarrow T$).

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Note added in proof

The nucleotide sequence data reported will appear in the EMBL Data Library under the accession number Z47081. The *Penicillium chrysogenum* homologue of the *A.nidulans acvA-ipnA* (dual promoter) intergenic region contains seven PacC consensus sites. Two of these (of the GCCAAG type) are clustered in a position approximating that of the *A.nidulans* ipnA2 site (relative to the isopenicillin *N*-synthetase coding region).