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# Activation of the *Aspergillus* PacC transcription factor in response to alkaline ambient pH requires proteolysis of the carboxy-terminal moiety

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Extremes of pH are an occupational hazard for many microorganisms. In addition to efficient pH homeostasis, survival effectively requires a regulatory system tailoring the syntheses of molecules functioning beyond the cell boundaries (permeases, secreted enzymes, and exported metabolites) to the pH of the growth environment. Our previous work established that the zinc finger PacC transcription factor mediates such pH regulation in the fungus *Aspergillus nidulans* in response to a signal provided by the products of the six *pal* genes at alkaline ambient pH. In the presence of this signal, PacC becomes functional, activating transcription of genes expressed at alkaline pH and preventing transcription of genes expressed at acidic pH. Here we detect two forms of PacC in extracts, both forming specific retardation complexes with a PacC-binding site. Under acidic growth conditions or in acidity-mimicking *pal* mutants (defective in ambient pH signal transduction), the full-length form of PacC predominates. Under alkaline growth conditions or in alkalinity-mimicking *pacC<sup>c</sup>* mutants (independent of the ambient pH signal), a proteolyzed version containing the amino-terminal ~40% of the protein predominates. This specifically cleaved shorter version is clearly functional, both as an activator for alkaline-expressed genes and as a repressor for acid-expressed genes, but the full-length form of PacC must be inactive. Thus, PacC proteolysis is an essential and pH-sensitive step in the regulation of gene expression by ambient pH. Carboxy-terminal truncations, resulting in a gain-of-function (*pacC<sup>c</sup>*) phenotype, bypass the requirement for the *pal* signal transduction pathway for conversion of the full-length to the proteolyzed functional form.

[Key Words: Transcriptional activation; transcriptional repression; proteolysis; DNA binding; signal transduction; pH]

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In common with many microorganisms, the physiologically versatile ascomycete fungus *Aspergillus nidulans* grows over a very wide pH range. Its syntheses of permeases, secreted enzymes, and exported metabolites are tailored to the pH of its growth environment (Caddick et al. 1986; Shah et al. 1991; Espeso et al. 1993). This physiological response is mediated by the zinc finger transcription factor PacC, an activator for alkaline-expressed genes and a repressor for acid-expressed genes (Tilburn et al. 1995). Six genes, designated *palA*, *palB*, *palC*, *palF*, *palH*, and *palI*, are involved in transduction of an alkaline ambient pH signal, necessary for the conversion of

PacC from an inactive to a functional form (Caddick et al. 1986; Arst et al. 1994; Tilburn et al. 1995). How the organism senses ambient pH and how alkaline ambient pH effects conversion of PacC from its inactive form to a functional activator/repressor are two fascinating, as yet unresolved, problems.

Here we address the second of these problems and show that two forms of PacC are detectable in *Aspergillus* extracts, both having PacC DNA-binding specificity. Under acidic growth conditions or in *pal* mutants defective in pH signal transduction (resulting in an acidity-mimicking phenotype), the full-length form of PacC predominates. At alkaline ambient pH or in gain-of-function *pacC<sup>c</sup>* mutants independent of pH signal

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transduction (resulting in an alkalinity-mimicking phenotype), a specifically proteolysed form is favored. In conjunction with mutational evidence (Tilburn et al. 1995), these results strongly support a model in which the carboxy-terminal region negatively modulates PacC function, rendering the full-length form inactive, and that a pH-sensitive step in conversion of PacC to a functional form is removal of the carboxy-terminal ~60% of the protein.

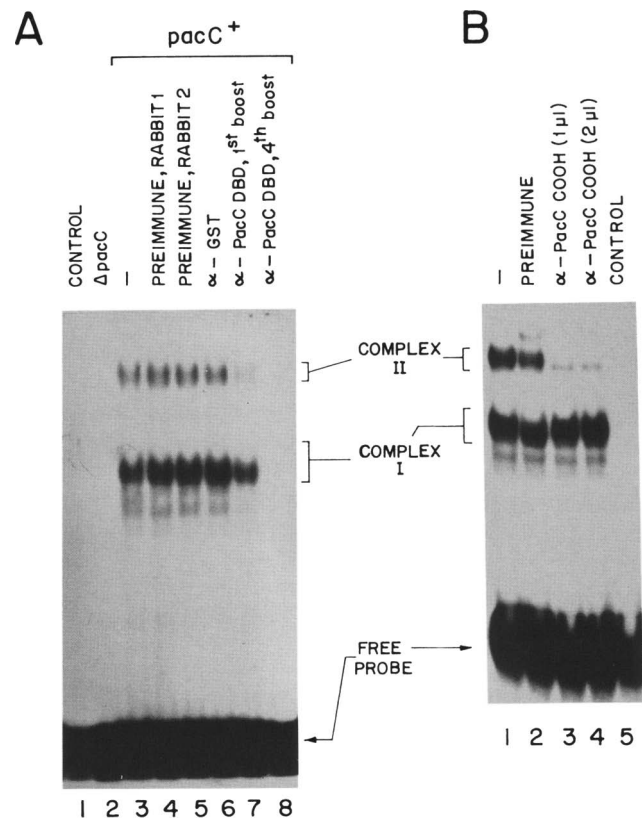
## Results

*The PacC protein can be detected in A. nidulans extracts and exists in two forms*

Pérez-Esteban et al. (1993) reported sequence-specific binding of a protein present in *A. nidulans* extracts to a region of the *ipnA* (isopenicillin N synthetase structural gene) promoter in which deletion analysis indicated the presence of a positive element required for full transcriptional activity. This region contains three in vitro PacC-binding sites (consensus 5'-GCCARG-3'), and PacC is a transcriptional activator of *ipnA* (Espeso et al. 1993; Tilburn et al. 1995). Preliminary experiments (not shown) correlated conclusively the presence of one (or more) PacC-binding sites with the formation of retardation complexes using an *A. nidulans* protein fraction enriched in the DNA-binding activity detected by Pérez-Esteban et al. (1993).

To confirm PacC-binding specificity of the protein detected in extracts, we used a 31-bp double-stranded oligonucleotide containing the high affinity *ipnA2* PacC-binding site (see Tilburn et al. 1995). Crude extracts formed two retardation complexes with this probe (Fig. 1A, lane 3; Fig. 1B, lane 1). Competition experiments with the wild type and a mutant 31-bp double-stranded oligonucleotide having a site-destroying A → T substitution in position 4 of the *ipnA2* core consensus (Tilburn et al. 1995) demonstrated that both complexes have PacC-binding specificity (data not shown). This conclusion was reinforced further by competition experiments using the lower affinity sites *ipnA1* and *ipnA3* (data not shown).

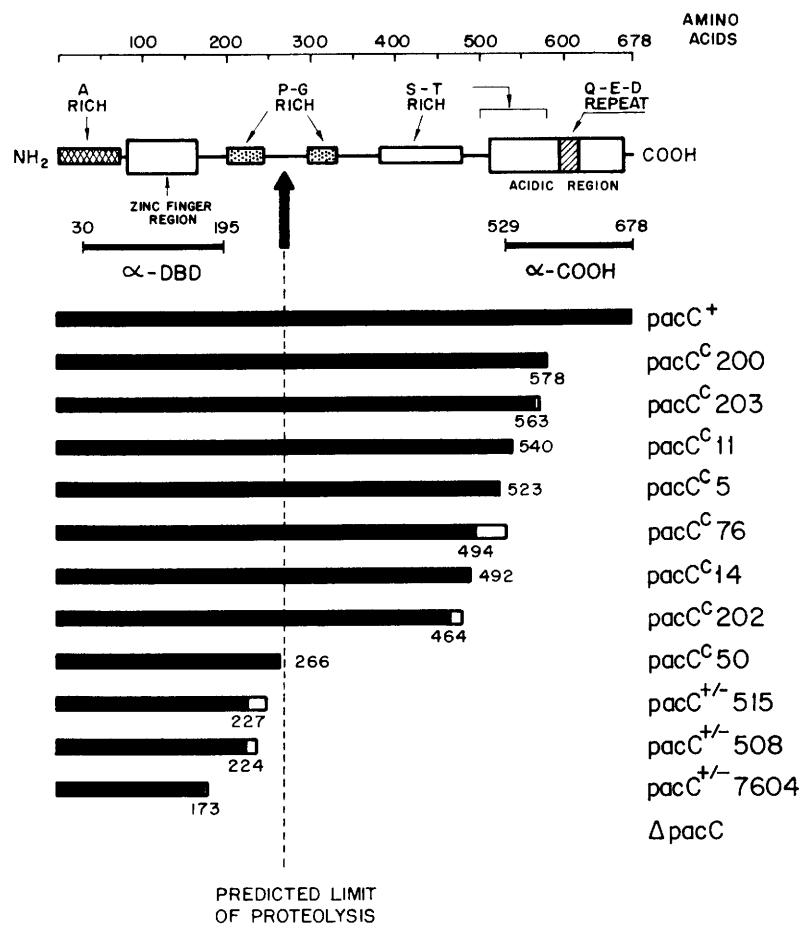
To test whether the protein components of the high mobility retardation complex (complex I) and/or the low mobility complex (complex II) contain PacC antigenic determinants, we included in binding reactions antisera raised against either a glutathione S-transferase (GST)::PacC(30–195\*) fusion protein, which contains the zinc finger DNA-binding domain (DBD), or against a GST::PacC(529–678) fusion protein containing the 150 PacC carboxy-terminal residues. GST::PacC(30–195\*) antiserum completely prevented the formation of both complexes I and II, whereas GST::PacC(529–678) antiserum largely prevented the appearance of complex II without affecting complex I formation (Fig. 1A, 1B). Control anti-GST antiserum had no effect. Thus, the protein components of both complexes I and II contain antigenic determinants in common with the PacC DBD, but only that of complex II contains antigenic determinants in common with the PacC carboxyl terminus. The effect of



**Figure 1.** PacC protein from *Aspergillus* extracts forms two retardation complexes with its cognate *ipnA2* site. Antiserum against the DBD prevents the formation of both complexes, whereas antiserum against the carboxy-terminal acidic region (COOH) prevents only the appearance of the low mobility complex. See Fig. 2 for position of fragments of the PacC protein separately overexpressed in *E. coli* as fusions to GST. The GST fusion proteins were used to immunize rabbits. (A) EMSA using a crude *Aspergillus* protein extract (5  $\mu$ g) and  $^{32}$ P-labeled synthetic *ipnA2* site (see Fig. 6 of Tilburn et al. 1995) as a probe. Inclusion of anti-DBD serum (2  $\mu$ l) weakly (first boost) or completely (fourth boost) prevents the formation of both complexes, whereas inclusion of two preimmune sera or a high titer anti-GST antiserum (a rabbit immunized with GST) had no effect. ( $\Delta$ *pacC*) A control reaction carried out with an equal amount of protein extract from a strain deleted for the entire *pacC*-coding region (Tilburn et al. 1995). (–) A control reaction in the absence of antisera. A (Control) mock reaction in the absence of protein extract. (B) A similar assay using 5  $\mu$ g of protein extract and the indicated amounts of antiserum against the carboxy-terminal acidic region. (Preimmune) A reaction in which 2  $\mu$ l of this serum was included; – and control reactions were as in A.

GST::PacC(529–678) antiserum on complex II formation probably results from the inability of the antigen–antibody complex to enter the gel, as this fusion protein does not bind DNA.

Figure 2 shows diagrammatically the derived translation products of a number of *pacC* mutant alleles. With the double-stranded 31-mer wild-type *ipnA2* site as probe, neither complex was formed using extracts from a



**Figure 2.** Schematized PacC protein showing selected features and predicted translation products of mutant alleles (Tilburn et al. 1995). The portions of the protein remaining in mutants used in this work are indicated by solid bars, with open bar extensions denoting approximate lengths of frameshifted abnormal sequences. The number of the most carboxy-terminal residue of normal PacC sequence is shown, either at the end of the bar (chain termination alleles) or below the end of the solid bar (frameshift alleles). Also shown are the positions of fragments present in fusion proteins used to raise antibodies and the predicted limit of proteolysis.

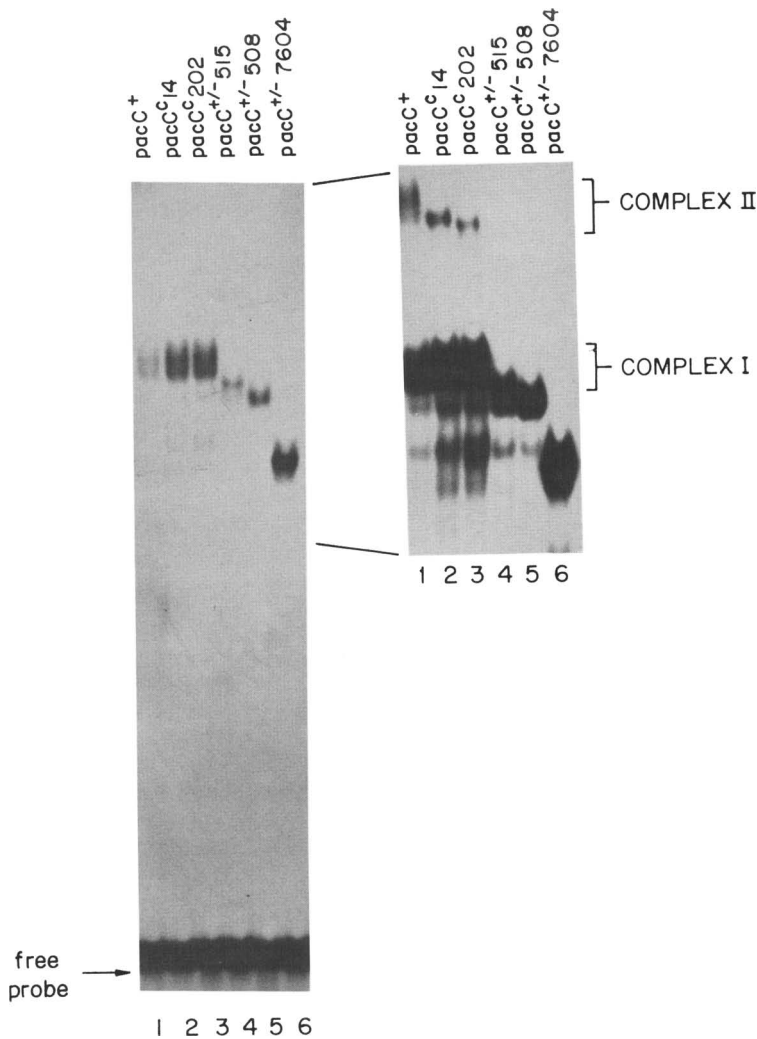
null mutant ( $\Delta pacC$ ) deleted for the entire coding region (Fig. 1A, lane 2). The gain-of-function mutations  $pacC^{C14}$  and  $pacC^{C202}$ , resulting in conceptual PacC translation products of 52,263 and 50,848 daltons, respectively, do not affect the mobility of complex I but increase the mobility of complex II to an extent consistent with the relative sizes of their predicted mutant proteins (Fig. 3). The partial loss-of-function frameshift mutations  $pacC^{+/-515}$ ,  $pacC^{+/-508}$ , and  $pacC^{+/-7604}$ , resulting in conceptual translation polypeptides of 26,756, 25,280, and 19,361 daltons, respectively, each result in a single complex whose mobility exceeds that of complex I commensurate with the decrease in predicted size of the PacC product (Fig. 3). PacC-binding specificity for all complexes formed with  $pacC$  mutant extracts was confirmed by the complete absence of complex formation when the mutant probe with A  $\rightarrow$  T in position 4 of the PacC core consensus was used (data not shown).

The mobilities of the complexes using  $pacC^{+/-7604}$ ,  $pacC^{+/-508}$ , and  $pacC^{+/-515}$  extracts and of complex II using  $pacC^{C202}$  and  $pacC^{C14}$  extracts (Fig. 3) are linear with  $\log_{10}$ -predicted relative molecular mass, and the wild-type complex II is only slightly more mobile than predicted for the full-length (678 residue) wild-type PacC (possibly reflecting loss of linearity in the high molecular

mass region of the gel). Using the linear  $\log_{10}$  plot derived using mutant extracts, a molecular mass of  $\sim 29$  kD can be interpolated for the wild-type protein component of complex I.

*Full-length and truncated forms of PacC in A. nidulans extracts are detectable by Western blotting and alkaline ambient pH favors predominance of the truncated form, apparently at the expense of the full-length form*

As PacC mediates regulation of gene expression in response to ambient pH, the PacC content of extracts from a wild-type strain was monitored after growth at three different pH values. A Western blot using antiserum against the GST::PacC(30–195\*) fusion protein containing the DBD (Fig. 4A) shows that two forms of PacC differing substantially in size are detectable. The migration rate of the larger is that expected for the full-length form (and contains epitopes from the carboxyl terminus as well as from the DBD; see Fig. 6B, below), whereas the smaller migrates at a rate approximately consistent with the (probably more accurate) size interpolated for the protein component of complex I. Under acidic growth conditions the full-length form predominates, and as the growth pH is raised, the amount of the smaller form



**Figure 3.** Effects of various *pacC* mutations truncating the protein product on mobility of complexes I and II. (left and right) Different exposures of an EMSA using the synthetic *ipnA2*-binding site and protein extracts from mycelia of strains carrying the indicated mutant alleles (see Fig. 2 for lengths of predicted polypeptides encoded by different alleles). Only the part of the longer exposure autoradiograph (right) necessary to detect formation of complex II in these conditions is shown. Mycelia of *pacC*<sup>+/–515</sup>, *pacC*<sup>c202</sup> and *pacC*<sup>c14</sup> strains were grown under acidic conditions, whereas those carrying the *pacC*<sup>+</sup>, *pacC*<sup>+/–508</sup> and *pacC*<sup>+/–7604</sup> alleles were grown under neutral conditions. [The *pacC*<sup>+</sup> strain was grown at neutral pH to facilitate simultaneous detection of the two complexes. Complex formation by the mutant extracts is pH insensitive (see text).]

increases progressively, apparently at the expense of the larger form, and comes to predominate. The behavior of PacC DNA-binding activity in electrophoretic mobility shift assays (EMSAs, Fig. 4B) mirrors the composition seen in the Western blot: At acidic growth pH the low mobility complex II predominates with progressive increases in (and predominance of) the high mobility complex I, apparently at the expense of complex II, as the growth pH is raised.

*PacC*<sup>c</sup> mutations mimicking alkalinity favor predominance of the smaller form of PacC irrespective of growth pH

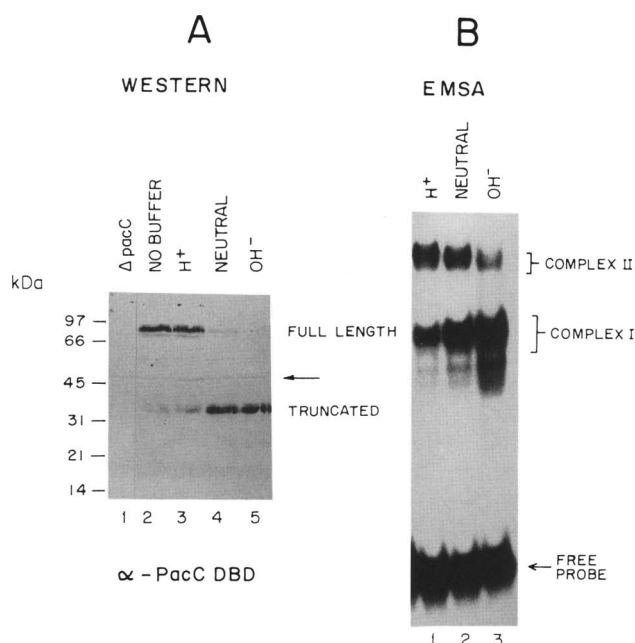
Figure 5 shows that alkalinity-mimicking, gain-of-function *pacC*<sup>c5</sup>, *pacC*<sup>c14</sup> and *pacC*<sup>c202</sup> mutations strongly increase levels of complex I and decrease levels of complex II under both acidic and alkaline growth conditions. For both *pacC*<sup>c14</sup> and *pacC*<sup>c202</sup>, levels of complex I are elevated irrespective of growth pH, whereas the phenotypically less extreme (Caddick et al. 1986) *pacC*<sup>c5</sup> mutation allows an increase in response to alkaline growth

pH. It is unlikely that the region between *pacC*<sup>c14</sup> (a stop in codon 493) and *pacC*<sup>c5</sup> (a stop in codon 524) is involved in the ambient pH response as *pacC*<sup>c11</sup> (a stop in codon 541; see Fig. 2) and *pacC*<sup>c200</sup> (a frameshift in codon 578, resulting in chain termination after a further threonine) lead to pH-independent, elevated levels of complex I (data not shown). Levels of complex-forming PacC are also independent of growth pH in a *pacC*<sup>+/–508</sup> strain.

The predominance of complex I formed with *pacC*<sup>c</sup> extracts correlates with a predominance of the smaller form of PacC seen in Western blots (Fig. 6). Using acidic growth conditions (where the full-length form of PacC predominates in wild-type extracts) and antibodies against the fusion protein containing the DBD, levels of full-length PacC were below limits of detection in extracts of a *pacC*<sup>c203</sup> (see Fig. 2) strain (Fig. 6A), as well as in extracts of *pacC*<sup>c14</sup> and *pacC*<sup>c202</sup> strains (data not shown). In contrast, levels of the smaller form of PacC were substantially elevated in extracts of these *pacC*<sup>c</sup> strains (Fig. 6A,C,D).

Just as no mobility differences were found between





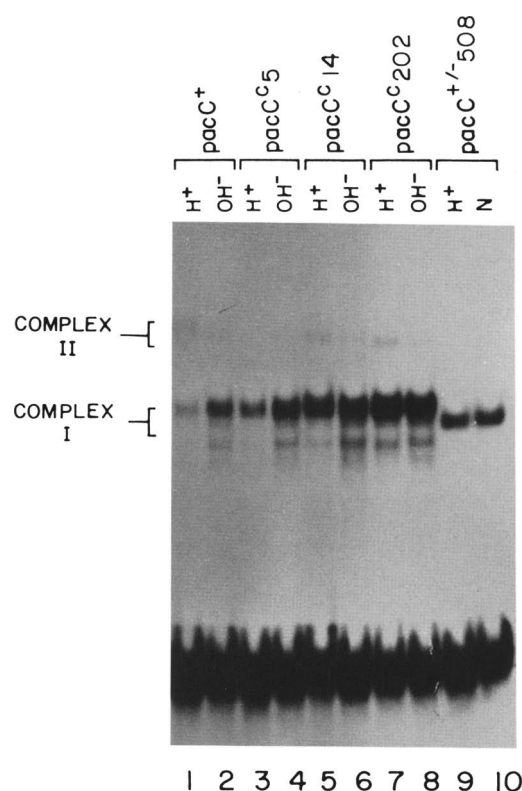
**Figure 4.** The relative proportions of the two forms of PacC correlate with ambient pH. (A) Western blot analysis of protein samples (50 µg) from different mycelial extracts, using antiserum against the PacC DBD (see Fig. 1). The positions of the full-length and truncated *pacC* products are indicated at right. An arrow marks the position of a nonspecific cross-reacting band present in all extracts, including that from a strain entirely deleted for *pacC*. This band serves as a loading control. The extracts used were from mycelia grown under acidic (H<sup>+</sup>) (initial pH 5.6, final pH 5.4), neutral (initial pH 6.8, final pH, 6.4), or alkaline (OH<sup>-</sup>) (initial pH, 7.9, final pH 7.1) conditions. The no buffer extract was from mycelia grown in PPB containing 3% (wt/vol) sucrose. Sucrose utilization in the absence of high buffering capacity results in strong acidification of the external medium. Therefore, for most purposes, these conditions, which show that high levels of phosphate ions present in acidic growth cultures are without effect, are equivalent to acidic conditions. (B) EMSA using the synthetic ipnA2-binding site and crude protein extracts (13 µg) from mycelia grown under the three different conditions of ambient pH described for A.

complex I formed using wild-type extracts and complex I formed using *pacC<sup>c</sup>* extracts (Figs. 3 and 5), no mobility differences were found between the wild-type smaller PacC form and those of *pacC<sup>c</sup>14*, *pacC<sup>c</sup>202*, or *pacC<sup>c</sup>203* strains using SDS-PAGE (Fig. 6C). In contrast, just as *pacC<sup>+/−</sup>* mutations lead to retardation complex mobilities greater than that of wild-type complex I (Figs. 3 and 5), the *pacC<sup>+/−</sup>515* (Fig. 6C), *pacC<sup>+/−</sup>508* (Fig. 6D), and *pacC<sup>+/−</sup>7604* (Fig. 6D) mutations increase PacC mobility in SDS-PAGE to extents correlated with predicted translation product sizes.

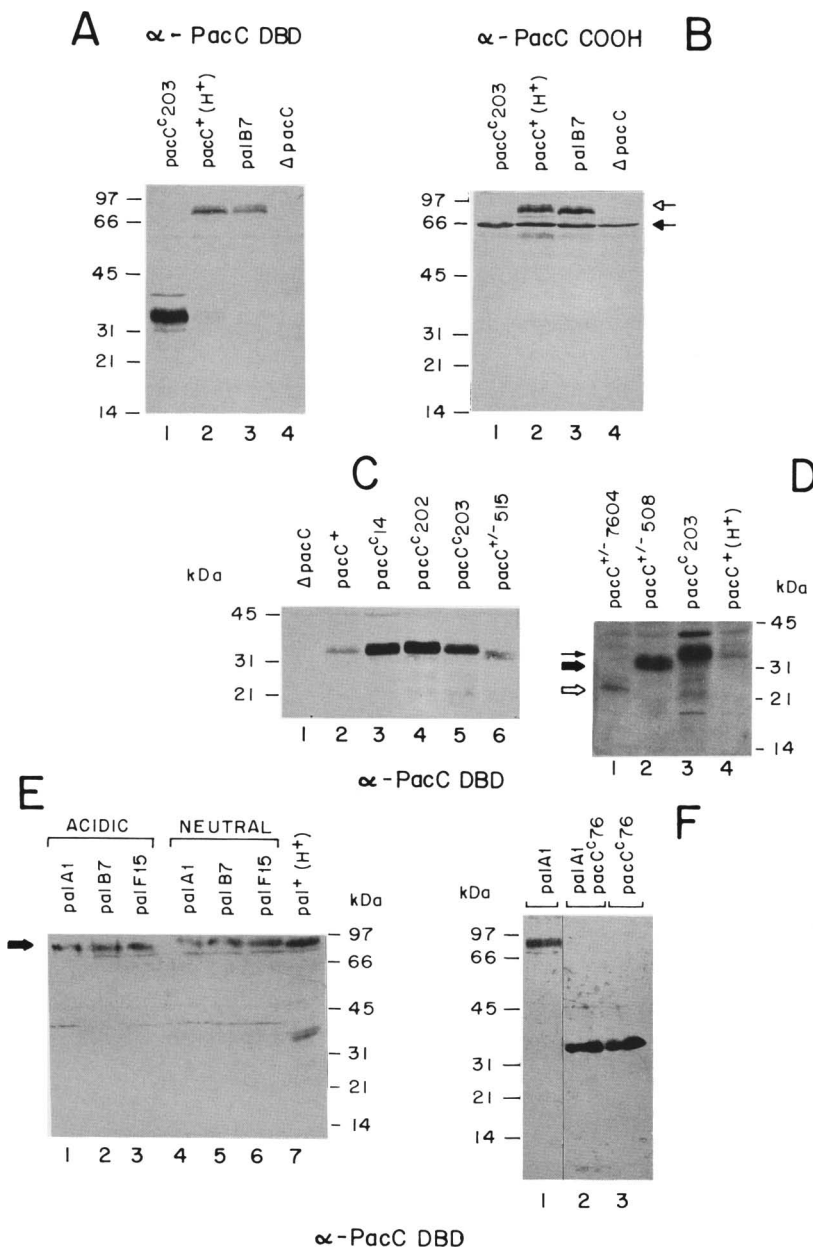
*pal* mutations mimicking acidic growth conditions and preventing alkaline ambient pH signal transduction largely prevent the appearance of the smaller form of PacC

Whereas wild-type extracts contain detectable amounts of the smaller form of PacC even under acidic growth

conditions (Figs. 4A and 6A,E), no smaller form of PacC is detectable by Western blotting of extracts of *palA1*, *palB7* or *palF15* strains even under neutral growth conditions (Fig. 6E). (Strains carrying acidity-mimicking *pal* mutations do not grow at alkaline pH [Caddick et al. 1986; Arst et al. 1994], and it is therefore not possible to monitor PacC under growth conditions optimal for production of the smaller form in the wild type.) The levels of full-length PacC approximate that of an acid-grown wild type (Fig. 6E). EMSA results (Fig. 7) are consistent with the Western blots: Complex II clearly predominates, although the greater sensitivity allows visualization of diffuse bands in the complex I region. Cross-competition experiments (not shown) demonstrate that the



**Figure 5.** Mutations mimicking alkalinity result in higher levels of complex I-forming activity irrespective of growth pH. The EMSA shows complex I formation using the synthetic ipnA2 site and extracts from mycelia carrying the *pacC<sup>+</sup>* allele or various gain-of-function alleles (*pacC<sup>c</sup>5*, *pacC<sup>c</sup>14* or *pacC<sup>c</sup>202*), which encode proteins with different truncations at the carboxyl terminus, as indicated in Fig. 2. *pacC<sup>+/−</sup>508* is a partial loss-of-function mutation. Its phenotype is acidity-mimicking, although less so than *pal* mutations [Tilburn et al. 1995]. H<sup>+</sup>, N, and OH<sup>-</sup> indicate acidic, neutral, and alkaline growth conditions, respectively. The exposure time was chosen to maximize differences in complex I-forming activity. Although this exposure allows detection of high mobility complex II formed by *pacC<sup>+</sup>* (H<sup>+</sup>) acidic extracts, it is too short to show the truncated complex II formed by extracts from gain-of-function mutants, in which nearly all the PacC-binding activity is in complex I [for complex II, see Fig. 3].

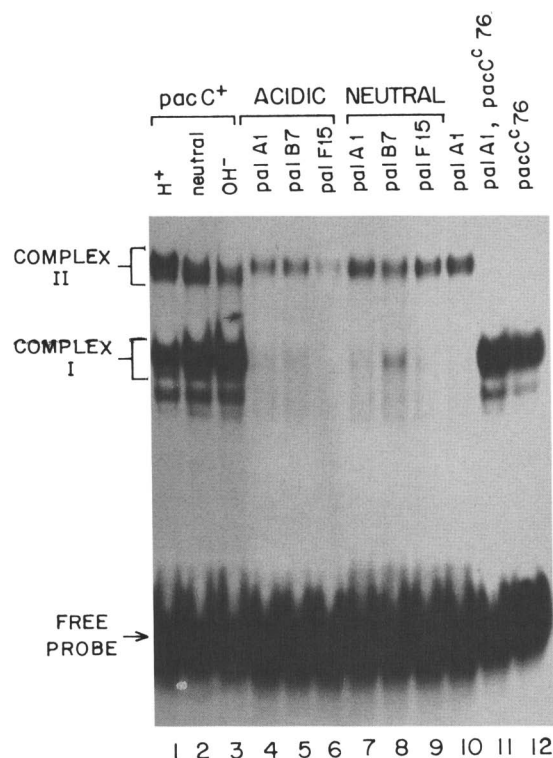


**Figure 6.** Western analysis of wild-type and mutant PacC protein forms in extracts. Fifty micrograms of protein extracts, corresponding to different growth conditions for wild-type or mutant strains, as indicated, was analyzed by Western blotting using antisera (see Fig. 1) against the PacC DBD (A and C–F) or against the PacC carboxy-terminal acidic region (B).  $\Delta pacC$  indicates a protein extract from a strain deleted for the entire *pacC*-coding region (Tilburn et al. 1995). Incubations with primary antisera were made in the presence of an excess of protein from the  $\Delta pacC$  extract (see Materials and methods) to prevent the appearance of nonspecific cross-reacting bands, which were also obtained with preimmune sera (data not shown). Nevertheless, in certain blots it is still possible to detect such bands, which can be identified because they are also present in the  $\Delta pacC$  lane. These bands are useful as loading controls. (A,B) All extracts were from mycelia grown in acidic conditions. (B) It should be noted that proteins used for immunization are fusions to GST, and the antisera specifically recognize an *A. nidulans* protein (probably an endogenous GST), which serves as loading control. This protein is also recognized by anti-GST antiserum but not by preimmune serum (data not shown). The amount of this antigen present in the  $\Delta pacC$  extract is insufficient for complete removal of this band, which has been therefore eliminated in most blots by including an excess of purified GST in the primary antisera incubation. Deliberately, however, this band has not been fully removed in this blot, where it is indicated by a solid arrow. Note that this protein, in contrast to the PacC band (open arrow), is present in all lanes (including  $\Delta pacC$ ). (C,D) Acidic growth conditions were used. Only the relevant portions of the gels, showing the processed, complex I-forming version of PacC (C) as well as differences in its mobility due to two *pacC*<sup>+/–</sup> mutations (D) are presented. In D, deliberate overexposure enabled detection of the severely truncated *pacC*<sup>+/–</sup>7604 product (Figs. 2 and 3). The positions of the proteolyzed PacC form (thin arrow), whose mobility is unaffected but whose levels are greatly elevated by the *pacC*<sup>C203</sup> mutation, and the PacC forms present in the *pacC*<sup>+/–</sup>508 (thick solid arrow) and *pacC*<sup>+/–</sup>7604 (thick open arrow) strains are indicated. (E) The full-length PacC version is indicated by a thick solid arrow. Growth conditions were as indicated. (F) Neutral growth conditions were used.

PacC-binding consensus is necessary for the appearance of these bands.

It should also be noted that the effects of acidity-mimicking *pal* mutations seen in Figures 6 and 7 are more extreme than the effects of acid growth conditions for the wild type seen in Figure 4 and that the effects of alkalinity-mimicking *pacC*<sup>C</sup> mutations seen in Figures 3, 5, 6, and 7 are more extreme than the effects of alkaline

growth conditions for the wild type seen in Figure 4. This reflects the relatively modest degrees of acidity (final pH 5.4) and alkalinity (final pH 7.1) in culture conditions for the experiment in Figure 4, whereas the mutant phenotypes are equivalent to the effects of more extreme acid or alkaline pH values. The response to ambient pH apparently occurs as a continuum rather than by discrete steps.



**Figure 7.** *pal* mutant extracts are defective in the formation of complex I. PacC ipnA2-binding activity in extracts from *pal* mycelia grown under acidic or neutral conditions. Binding reactions contained 7.5  $\mu$ g of protein extracts. Also shown is the epistasis of a *pacC*<sup>c</sup> gain-of-function allele (*pacC*<sup>c76</sup>) to a *palA* mutation. Mycelia grown at neutral pH were used for the experiment shown in lanes 10–12.

*An alkalinity-mimicking *pacC*<sup>c</sup> mutation is epistatic to an acidity-mimicking *pal* mutation with respect to PacC composition of extracts*

Mutations in *pacC* are epistatic to those in the six *pal* genes of the pH signal transduction pathway in every combination tested (Caddick et al. 1986; Arst et al. 1994; Tilburn et al. 1995). This epistasis, reported previously for growth characteristics and enzyme production, also extends to PacC processing. Figure 6F shows that *pacC*<sup>c76</sup> is epistatic to *palA1* with respect to predominance of the smaller PacC form, a result mirrored by predominance of retardation complex I (Fig. 7).

*Conversion of the full-length form of PacC to the smaller form does not take place during extraction*

Exploiting the virtual absence of the shorter form of PacC in *palA1* strains (Fig. 7E,F) and the difference in mobilities of complex II between *palA1* and *pacC*<sup>c202</sup> strains (Figs. 3 and 7), we mixed equal amounts of mycelia of the two strains prior to extraction. This did not result in conversion of the complex II formed by the PacC from the *palA1* (*pacC*<sup>+</sup>) strain to the high mobility complex I (data not shown). Equally, incubating mixed

extracts from the two strains for 30 min at 37°C in the EMSA buffer had no effect (data not shown). Thus, the conversion of PacC from the larger to the smaller form is likely to occur *in vivo* rather than during *in vitro* manipulations.

## Discussion

### *The functional form of PacC is the smaller version*

Tilburn et al. (1995) presented a model, strongly buttressed by mutational evidence and Northern analysis, in which PacC is in a form functional both as an activator of alkaline-expressed genes and as a repressor of acid-expressed genes under alkaline growth conditions but is in an inactive form under acidic growth conditions. On the basis of mutational and immunological evidence and DNA-binding properties, we have identified two forms of PacC in extracts, the probable full-length (678 residue) 73-kD primary translation product and a smaller ~29-kD form, whose deduced carboxyl terminus is located at approximately residue 270 (vide infra). The larger form is the protein component of retardation complex II, and the smaller form is the protein component of retardation complex I. Although both forms contain the zinc finger region and bind DNA, compelling reasons indicate that the smaller version is the functional form. First, the smaller version predominates under alkaline growth conditions, whereas the larger version predominates under acidic growth conditions. Second, levels of the smaller version are substantially elevated, whereas the

**Table 1.** *Aspergillus* strains used in this study

Strain designation	Complete genotype
Wild type	<i>biA1</i>
$\Delta$ <i>pacC</i>	$\Delta$ <i>pacC::pyr4</i> <sup>+</sup> , <i>pabaA1</i> , <i>biA1</i> , <i>yA2</i>
<i>pacC</i> <sup>c5</sup>	<i>pacC</i> <sup>c5</sup> , <i>pabaA1</i>
<i>pacC</i> <sup>c11</sup>	<i>pacC</i> <sup>c11</sup> , <i>pantoB100</i>
<i>pacC</i> <sup>c14</sup>	<i>pacC</i> <sup>c14</sup> , <i>biA1</i>
<i>pacC</i> <sup>c76</sup>	<i>pacC</i> <sup>c76</sup> , <i>inoB2</i>
<i>pacC</i> <sup>c200</sup>	<i>pacC</i> <sup>c200</sup> , <i>gatA2</i> , <i>pantoB100</i> , <i>yA2</i>
<i>pacC</i> <sup>c202</sup>	<i>pacC</i> <sup>c202</sup> , <i>pabaA1</i>
<i>pacC</i> <sup>c203</sup>	<i>pacC</i> <sup>c203</sup> , <i>pabaA1</i> , <i>pantoB100</i> , <i>bwA1</i>
<i>pacC</i> <sup>+/–508</sup>	<i>pacC</i> <sup>+/–508</sup> , <i>pacC</i> <sup>c5</sup> , <i>pabaA1</i> , <i>yA2</i>
<i>pacC</i> <sup>+/–515</sup>	<i>pacC</i> <sup>+/–515</sup> , <i>pacC</i> <sup>c5</sup> , <i>pantoB100</i> , <i>yA2</i>
<i>pacC</i> <sup>+/–7604</sup>	<i>pacC</i> <sup>+/–7604</sup> , <i>pacC</i> <sup>c76</sup> , <i>inoB2</i>
<i>pacC</i> <sup>c50</sup>	<i>pacC</i> <sup>c50</sup> , <i>lysA2</i>
<i>palA1</i>	<i>palA1</i> , <i>biA1</i> , <i>wA3</i>
<i>palB7</i>	<i>palB7</i> , <i>pabaA1</i>
<i>palF15</i>	<i>palF15</i> , <i>pantoB100</i> , <i>yA2</i>
<i>pacC</i> <sup>c76</sup> , <i>palA1</i>	<i>pacC</i> <sup>c76</sup> , <i>palA1</i> , <i>pabaA1</i> , <i>wA3</i>

Sequence changes for *pacC* mutations are given by Tilburn et al. (1995), with the exception of that for *pacC*<sup>c50</sup> (Rand 1978; C.R. Bailey and H.N. Arst, unpubl.), described here. It is an A  $\rightarrow$  T transversion at nucleotide 1757 and an amber mutation in codon 267.



full-length form is nearly undetectable in alkalinity-mimicking, carboxy-terminally truncated *pacC<sup>c</sup>* mutants, a gain-of-function class that bypasses the need for alkaline ambient pH signal transduction. Third, the smaller version is almost undetectable in acidity-mimicking *pal* mutants blocked in alkaline ambient pH signal transduction. Finally, we have determined recently that *pacC<sup>c</sup>50* is an amber mutation in codon 267 (Table 1; Fig. 2), very near to the deduced carboxyl terminus of the smaller version. *pacC<sup>c</sup>50* has a gain-of-function (*pacC<sup>c</sup>*) phenotype, strongly suggesting that the smaller version of PacC contains sufficient residues to be fully functional.

PacC proteolysis is almost certainly physiological because it does not occur during extraction or extract incubation, as might be expected if it were an artifact attributable, for example, to a protease under *pacC* positive control.

*Specific proteolysis is a pH-sensitive step in conversion of PacC to its functional form*

The lack of carboxy-terminal epitopes of PacC in the smaller version and the fact that *pacC<sup>c</sup>* mutations truncating up to 214 carboxy-terminal amino acids affect the mobility of complex II but do not affect mobility of complex I indicate that the processing event involved in conversion of the inactive full-length (678 residue) form of PacC to the smaller functional form involves considerable carboxy-terminal proteolysis, a conclusion consistent with data indicating that the amount of the smaller form of PacC increases at the expense of the larger form. Assuming that the amino terminus remains intact, the limit of proteolysis determined from the interpolated size of the protein component of the wild-type complex I would be at approximately residue 270. This limit appears precise because although after deliberate overexposure of EMSA autoradiographs, bands of higher mobility than complex I (presumably representing complexes with degradation products of the processed form of PacC) are visible, no bands of intermediate mobility between complex II and complex I have ever been detected (e.g., Fig. 3, right).

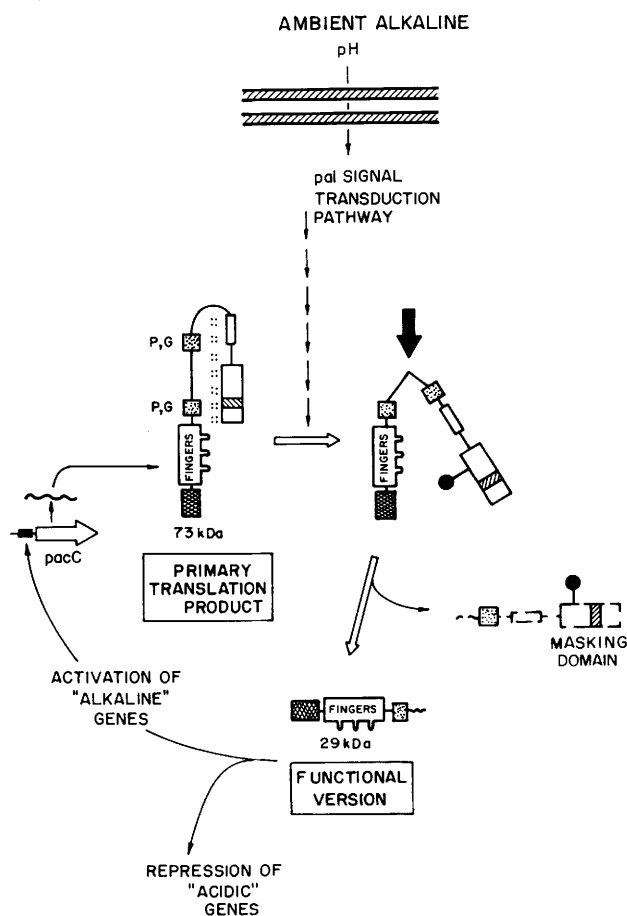
Because of the epistasis of *pacC<sup>c</sup>* mutations to extant acidity-mimicking *pal* mutations (Caddick et al. 1986; Arst et al. 1994), the PacC-processing proteolytic activity is very unlikely to be catalyzed by one of the six *pal* gene products of the pH signal transduction pathway. The presence of the proteolyzed form of PacC in extracts of a *palA1 pacC<sup>c</sup>76* double mutant (Figs. 6F and 7) definitively establishes that *palA* does not encode the protease in question. Nevertheless, mutations leading to loss of the protease should at least have a *pal* mutant phenotype. If the protease has a role in addition to PacC proteolysis, mutations leading to its loss might not have been recoverable in previous genetic screens. It is worth noting that processing of the NF- $\kappa$ B p50 precursor, which is also activated by carboxy-terminal proteolysis (vide infra), occurs through the ubiquitin-mediated protein degradation pathway (Fan and Maniatis 1991; Palombella et al. 1994;

for review, see Ciechanover 1994) and that disruption of yeast genes encoding components of the 26S protease of this pathway (Ghislain et al. 1993) or *UBA1*, encoding an E1 ubiquitin-activating enzyme (McGrath et al. 1991), is lethal. It therefore seems likely that the *pal* pH signal transduction pathway mediates a modification of the PacC primary translation product essential for subsequent proteolytic processing (see Fig. 8). The processed carboxyl terminus of NF- $\kappa$ B is rapidly degraded (Fan and Maniatis 1991). We note that residues 539–550 of PacC (Tilburn et al. 1995) constitute a PEST-rich (Rogers et al. 1986) sequence.

If the function of a protein is masked by intramolecular interactions, proteolytic removal of the masking region is a possible mechanism to convert the protein to a functional form. Examples include activation of two  $\sigma$ -factors mediating cell-type-specific transcription during sporulation in *Bacillus subtilis* (for review, see Losick and Stragier 1992; Haldenwang 1995). Twenty-nine and 20 amino-terminal amino acids, respectively, of pro- $\sigma^E$  (LaBell et al. 1987) and pro- $\sigma^K$  (Lu et al. 1990) are removed in developmentally regulated fashion to produce active  $\sigma$ -factors. The amino-terminal region of pro- $\sigma^K$  negatively modulates DNA binding (Dombroski et al. 1993). Proteolytic activation has also been demonstrated for certain eukaryotic transcription factors. The carboxyl terminus of the 105-kD precursor of NF- $\kappa$ B is removed to yield a 50-kD active form. This carboxyl terminus prevents nuclear localization, DNA binding, heterodimerization, and its own proteolytic removal (Ghosh et al. 1990; Kieran et al. 1990; Blank et al. 1991; Fan and Maniatis 1991; Henkel et al. 1992; Rice et al. 1992; Palombella et al. 1994). The activity of the sterol regulatory element-binding protein SREBP-1 is regulated in a formally similar, albeit mechanistically distinct way: The carboxyl terminus anchors the 125-kD primary translation product to the endoplasmic reticulum membrane and its cholesterol-dependent cleavage allows translocation of the 68-kD amino terminus into the nucleus (Wang et al. 1994). Here we have found that in the absence of the alkaline ambient pH signal, the wild-type (but not truncated *pacC<sup>c</sup>* mutant) carboxyl terminus of PacC prevents proteolytic processing.

*A model for the role of PacC proteolysis in the pH regulatory circuit*

Figure 8 presents a model. The 73-kD PacC primary translation product is held in an inactive form through intramolecular interactions (denoted by double colons) with the carboxyl terminus. At alkaline ambient pH, the *pal* gene products of the pH signal transduction pathway introduce a modification (indicated as a solid circle with line attachment) disrupting the intramolecular interactions and allowing proteolysis to a limit located between the two proline/glycine-rich regions (as indicated by a thick black arrow). Proteolytic removal of the carboxyl terminus would convert PacC into a functional form, capable of activating transcription of alkaline-expressed genes and preventing transcription of acid-expressed



**Figure 8.** A model for the role of PacC proteolysis in pH regulation.

genes. Autogenous regulation of *pacC* (Tilburn et al. 1995) would lead to more primary translation product at alkaline pH, which in turn would be processed to yield yet more functional version. By truncation of a portion of the carboxyl terminus, *pacC<sup>c</sup>* gain-of-function mutations would reduce intramolecular interactions sufficiently to allow proteolysis even in the absence of the *pal* signal pathway modification, resulting in continuous synthesis of functional PacC irrespective of ambient pH. Loss-of-function mutations in any of the signal pathway *pal* genes would prevent the modification disrupting the intramolecular interaction and result in perpetually inactive PacC.

## Materials and methods

### *A. nidulans* strains and growth conditions at different ambient pH

*A. nidulans* strains used in this work are listed in Table 1. Mycelia for protein extraction were grown in appropriately supplemented penicillin production broth (PPB, Espeso and Peñalva 1992), which allows vigorous growth and high biomass yields at any pH within the range used here. To achieve the different growth conditions with respect to ambient pH, 100 mM phosphate and 200 mM Na<sup>+</sup> were included in PPB. Alkaline broth

was made by including these ions as 100 mM disodium hydrogen phosphate (resulting in initial pH 7.9–8.0). Neutral broth (initial pH 6.8) was made by including 50 mM disodium hydrogen and 50 mM monosodium dihydrogen phosphate, together with 50 mM NaCl. Acidic broth (initial pH 5.6) was made by including 100 mM monosodium phosphate plus 100 mM NaCl.

### Preparation of GST fusion proteins with different regions of PacC

Plasmid pGEX-PacC, which drives expression in *Escherichia coli* of a GST::PacC(30–195\*) fusion protein containing the three PacC zinc fingers plus flanking amino acid sequences, has been described (Tilburn et al. 1995). A plasmid expressing a GST::PacC(529–678) protein was constructed by inserting a 0.65-kb *Bgl*III (filled in with Klenow)–*Eco*RI fragment (obtained from a cDNA *pacC* clone) between the *Sma*I and *Eco*RI sites of pGEX-2T (Pharmacia). GST fusion proteins were expressed in *E. coli* and purified by glutathione affinity chromatography in the presence of 0.5 M NaCl as described (Tilburn et al. 1995).

### Preparation of *A. nidulans* protein extracts

Conidiospores of the various strains were inoculated at a density of  $1 \times 10^6$  to  $2 \times 10^6$  spores/ml in 200 ml of PPB (at the appropriate initial pH). Shaken cultures were grown for 24 hr at 37°C. Protein was extracted from washed mycelia as described previously (Pérez-Esteban et al. 1993), with the following modification: After the final ammonium sulfate precipitation, the resulting protein pellet was resuspended in 2–3 ml of buffer A [25 mM HEPES–KOH at pH 7.9, 20% (vol/vol) glycerol, 5 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 1 mM DTT, 2 μM pepstatin, and 0.6 mM leupeptin] containing 100 mM KCl (buffer A<sub>100</sub>). Residual ammonium sulfate was eliminated by filtration through a Pharmacia PD-10 column. Protein extracts, divided into small aliquots, were stored at –80°C. Protein concentrations were determined by the Bradford (1976) assay.

### EMSAs

The synthetic wild-type and mutant (A<sub>4</sub> → T) ipnA2 PacC-binding sites were obtained after annealing of the corresponding single-stranded oligonucleotides as described (Tilburn et al. 1995). A range of 30–100 ng of the ipnA2 double-stranded probe was terminally labeled with Klenow polymerase and [ $\alpha$ -<sup>32</sup>P]dCTP (3000 Ci/mmol) in excess of the other (cold) dNTPs. Binding reactions, essentially as described previously (Pérez-Esteban et al. 1993), were made with 0.3 ng of the ipnA2 probe (usually 30K cpm) and 3–7.5 μg of the corresponding *Aspergillus* protein fraction (see figure legends). Reaction mixtures (20 μl) contained 25 mM HEPES–KOH (pH 7.9), 50 mM KCl, 5 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 1 mM DTT, 2 μg of poly[d(I–C)]/poly[d(I–C)], and 21% (vol/vol) glycerol. Proteins and DNA were allowed to equilibrate for 30 min at 0°C. Protein–DNA complexes were resolved in nondenaturing 8% polyacrylamide gels in 0.5× TBE, which were run at 4°C and 10 mA, dried, and either autoradiographed or used to quantify the label in each band with a PhosphorImager (Molecular Dynamics).

### Preparation of antibodies and Western analysis

Polyclonal antisera against GST::PacC(30–195\*) or GST::PacC(529–678) were raised in rabbits, using affinity-purified fusion proteins. The first immunizations were made by subcutaneous injections of fusion proteins with complete Freund's adjuvant. Booster injections (three to each rabbit) were given every

2 weeks, the first of which was made with incomplete Freund's adjuvant. Sera were collected 2 weeks after the last immunization. As a control, antiserum against purified GST was also raised. Amounts of 1–2  $\mu$ l of the unpurified antisera were included in the binding reactions, together with the protein extracts. For Western analysis, protein samples (50  $\mu$ g) were run in Laemmli SDS–polyacrylamide gels and electrophoretically transferred at 300–400 mA for 2 hr to Hybond ECL nitrocellulose membrane (Amersham) in 25 mM Tris, 193 mM glycine, 20% (vol/vol) methanol by using a submerged blotter. Blots were calibrated by using prestained protein standards (Bio-Rad, low range). The efficiency of the transfers was tested by Ponceau S staining of the membranes. Nonspecific sites were blocked by incubation of the blots for at least 2 hr at room temperature in 5% (wt/vol) nonfat dry milk in 20 mM Tris-HCl (pH 7.5), and 500 mM NaCl (TBS). Then blots were incubated overnight at 4°C in TTBS [TBS plus 0.05% (vol/vol) Tween 20 with 1% (wt/vol) nonfat dry milk in the presence of the relevant specific antiserum, at a dilution of 1:5000 [antiserum against the PacC(30–195\*) fusion protein] or 1:4000 [antiserum against the PacC(529–678) fusion protein]. To reduce excessive background, the primary antibody cocktails were preincubated with 0.5 mg of a protein extract from the *A. nidulans*  $\Delta$ pacC strain for 1 hr at 37°C before being added to the blots. In addition, a prominent *A. nidulans* band cross-reacting with anti-GST antiserum (and therefore detected by antisera against the GST fusions) was subtracted after preincubation with an excess of purified GST. After the incubation with the primary antibody, blots were washed three times with TTBS and incubated for 1 hr at room temperature with a 1:4000 dilution of goat anti-rabbit secondary antibody (Sigma), conjugated to horseradish peroxidase. After three washes in TTBS and one wash in TBS, horseradish peroxidase activity was detected by using an Enhanced Chemiluminescence Detection Kit (Amersham), following the manufacturer's instructions. Blots were exposed to Kodak X-OMAT film for 5–30 sec.

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