Regulation of Gene Expression by Ambient pH in Filamentous Fungi and Yeasts

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HISTORICAL INTRODUCTION TO pH REGULATION

Many microorganisms, particularly if they are able to grow over a wide pH range, tailor gene expression to the pH of their growth environment. Genes whose expression is likely to be influenced by ambient pH include those involved in the provision of secreted enzymes, permeases, and exported metabolites, all of which must function at ambient pH, and probably also those involved in internal pH homeostasis. Observations suggesting that gene regulation can respond appropriately to ambient pH extend back more than half a century for bacteria (49, 50; for reviews, see references 46, 46a, 57, and 65) and at least a quarter of a century for fungi (25, 72, 89, 107, 123).

The groundwork for a systematic investigation of ambient pH regulation of gene expression in fungi was unknowingly laid by Gordon Dorn. Dorn (34, 35) selected a large number of mutations affecting acid and/or alkaline phosphatase in *Aspergillus nidulans* and found that they identified an unexpectedly large number of genes. He originally proposed that the phosphatases are hetero-oligomeric, a hypothesis that his subsequent biochemical studies failed to confirm. However, by monitoring both secreted acid phosphatase, an enzyme synthesized under acidic growth conditions, and secreted alkaline phosphatase, an enzyme synthesized under alkaline conditions, he contributed both a methodology and experimental material which were to be crucial to the study of pH regulation.

Our involvement in pH regulation dates back to the mid-1960s, when one of us (H.N.A.) was doing a Ph.D. at the University of Cambridge under the supervision of David Cove, who had selected a number of mutations conferring resistance to molybdate toxicity in *Aspergillus nidulans* in the hope that they would prove useful in the analysis of nitrate reductase and the molybdenum-containing cofactor it shares with other molybdo-enzymes. In the course of mapping these mutations, I used a number of Dorn's mutants. Unexpectedly, many of the mutations selected by Dorn resulted in resistance or hypersensitivity to molybdate toxicity (1, 4), a finding which was to remain enigmatic for the better part of two decades. The next pleiotropic effect emerged when it was found that Dorn's *pacC^c5* mutation reduced γ-aminobutyrate (GABA) transport (2). An apparently altered electrophoretic mobility (35) led to the mistaken proposal that *pacC* encodes a secreted acid phosphatase involved in the synthesis, membrane integration, or functioning of certain permeases, notably those for GABA and molybdate (2). The hypothesis was to be discarded, but GABA transport analysis has proved invaluable in investigating pH regulation.

Shortly afterward, Mark Caddick embarked on a Ph.D. His thesis (14) contains suggested likely roles for nearly every gene Dorn had identified (15, 17, 109). Reproducibility of the altered electrophoretic mobility attributed to *pacC^c5* proved elusive (14), and, from what is now known, any such altered mobility in a *pacC* mutant is likely to have resulted from an effect on glycosylation of the acid phosphatase (93, 131).

pH REGULATION IN *ASPERGILLUS NIDULANS*

pH Regulatory Domain

Identified *Aspergillus nidulans* genes subject to regulation by ambient pH can be classified into three categories: those encoding secreted enzymes, those encoding permeases, and those encoding enzymes involved in synthesis of exported metabolites. pH-regulated genes encoding secreted enzymes include *pacA* encoding acid phosphatase (15, 17), *xlnB* encoding a xylanase (75, 98), *abfB* encoding an α-L-arabinofuranosidase (54), and one or more genes encoding an acid phosphodiesterase(s) (16, 17), all expressed preferentially under acidic growth conditions, as well as *palD* encoding alkaline phosphatase (15, 17), *prtA* encoding an alkaline protease (62, 122, 128), and *xlnA* encoding a xylanase (75, 96, 98), all expressed preferentially under alkaline growth conditions. pH-regulated genes encoding permeases include *gabA* encoding a GABA transporter having an acidic pH optimum and expressed preferentially at acidic ambient pH (6, 17, 39, 59). Indirect evidence suggests that a molybdate permease might be synthesized preferentially under acidic conditions (4, 17). pH-regulated genes involved in synthesis of exported metabolites include the preferentially alkaline-expressed *acvA* encoding δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase and *ipnA* encoding isopenicillin N-synthase, the first two enzymes of penicillin biosynthesis (40, 42, 112, 121), as well as the preferentially acid-expressed *stcU* encoding a ketoreductase catalyzing the reduction of versicolorin A and participating in synthesis of the toxin sterigmatocystin, which, in certain other *Aspergillus* species, is a precursor of aflatoxins (9, 64). Additionally, one or more activities involved in the toxicity of aminoglycosides such as neomycin and kanamycin appears to be under ambient pH control such that toxicity is greatest under alkaline growth conditions (17). The *pacC* gene encoding the transcription factor mediating pH regulation is itself preferentially expressed at alkaline pH (122).

Formal Genetics of pH Regulation

Mutations affecting pH regulation fall mainly into two categories. The first is that of alkalinity-mimicking mutations which, irrespective of ambient pH, lead to a pattern of gene expression similar to that in the wild type grown under alkaline conditions. These mutations, for example, reduce GABA transport and acid phosphatase, increase alkaline phosphatase levels, and result in molybdate resistance and neomycin hypersensitivity (Table 1). The second category is that of acidity-mimicking mutations which, irrespective of ambient pH, lead to a pattern of gene expression similar to that shown by the wild type grown under acidic conditions and show the opposite phenotype to mutations in the first category (17, 122). For example, they prevent or reduce growth at alkaline pH, reduce alkaline phosphatase levels, increase GABA transport and acid phosphatase levels, and confer neomycin resistance and molybdate hypersensitivity (Table 1). In addition, a small number of mutations have a neutrality-mimicking phenotype irrespective of ambient pH in which the pattern of gene expression approximates that of the wild type grown at pH 6.5 or has a heterogeneous mixture of alkalinity-mimicking and acidity-mimicking characteristics (83).

pH regulation is mediated mainly by seven genes, *pacC*, *palA, palB, palC, palF, palH*, and *palI*, where mutations lead to either alkalinity, acidity or, occasionally, neutrality mimicry.

Acidity-, alkalinity- and neutrality-mimicking mutations have been obtained in *pacC* (17, 33, 39, 83, 95, 122). This

TABLE 1. Approximate plate test phenotypes of *A. nidulans* mutations affecting pH regulation[a]

Relevant genotype	Growth at 25°C	Growth at pH 8	Growth[b] in the presence of MoO_4^{2-}	Growth[c] in the presence of neomycin	Acid phosphatase staining[d]	Alkaline phosphatase staining[d]	GABA utilization[e] in *areA*[r] strain
pacC[c]	+++	+++	++	−	−	++	−
Wild type	+++	+++	+	+	+	+	−
pacC[f]	−	−	−	+++	+++	−	++
pacC^{+/−}, *palA*[−], *palB*[−], *palC*[−], *palF*[−], *palH*[−]	+++	−	−	+++	+++	−	++
palI[−], *palF*^{+/−}, *palH*^{+/−}	+++	+	−	+++	++	−	++

[a] Unless otherwise specified, growth is at 37°C on appropriately supplemented minimal medium at pH 6.5.
[b] Concentration range, 20 to 30 mM.
[c] Concentration range, 0.5 to 2 mg/ml.
[d] On medium lacking phosphate.
[e] As the sole nitrogen source. See reference 3.
[f] Growth scores take into account a reduction of growth on all media.

phenotypic heterogeneity reflects the more direct involvement of the *pacC* product (the key transcription factor, see below) in regulation of gene expression by ambient pH. The *pacC* acronym reflects the loss of acid phosphatase at acidic pH characteristic of the (numerous) alkalinity-mimicking gain-of-function class (34), denoted *pacC*[c] (although strictly speaking these mutations are "constitutive" only for expression of alkali-expressed genes and are "superrepressed" for acid-expressed genes irrespective of ambient pH [Fig. 1]). In contrast to *pacC*[c] mutations, which are partially dominant to the wild-type allele, mutations in the partial- and complete-loss-of-function class, denoted *pacC*^{+/−} and *pacC*[−], respectively, are mostly recessive

and lead to acidity mimicry. Neutrality-mimicking mutations (which lead to simultaneous expression of both "acidic" and "alkaline" genes) are denoted *pacC*[c/−].

In contrast to the above heterogeneity, only acidity-mimicking mutations have been obtained in *palA, palB, palC, palF, palH,* and *palI* (3, 17, 95, 122). Mutations in these six *pal* genes are in all cases recessive and hypostatic to (i.e., phenotypically masked or suppressed by) the vast majority of *pacC* mutations (3, 17, 95; L. A. Rainbow, J. Tilburn, and H. N. Arst, Jr., unpublished data). Null mutations in *pacC* and the six *pal* genes lead to strong acidity mimicry (31, 32, 90, 91, 122; H. J. Bussink, M. A. Peñalva, and H. N. Arst, Jr., unpublished data).
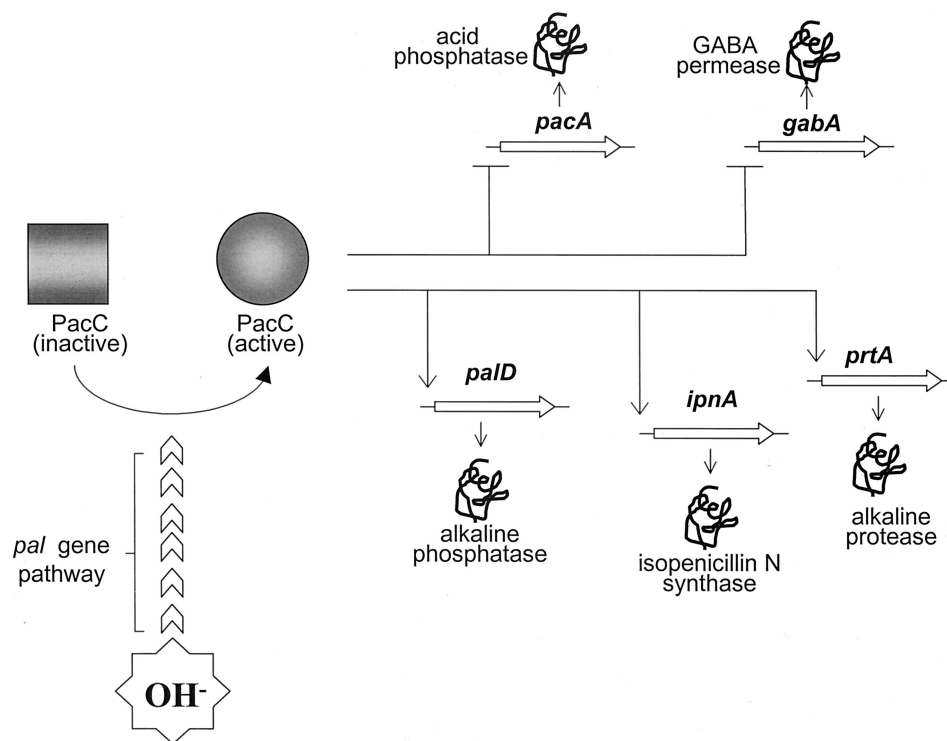
FIG. 1. Formal genetic model of pH regulation in *A. nidulans*. PacC is synthesized as an inactive form whose activation requires a signal transduced under alkaline pH conditions by the *pal* signaling pathway. The active form of PacC is a transcriptional repressor of acid-expressed genes, such as *pacA* and *gabA*, and a transcriptional activator of alkaline-expressed genes, such as *ipnA, prtA,* and *palD*. Mutations inactivating *pacC* (*pacC*[−] or *pacC*^{+/−}) or the *pal* signaling pathway lead to absence of expression of alkaline genes and derepression of acidic genes, which results in acidity mimicry. Gain-of-function *pacC*[c] mutations bypassing the *pal* signaling pathway (i.e., leading to active PacC at any ambient pH) result in permanent activation of alkaline genes and superrepression of acidic genes, which leads to alkalinity mimicry.

A

B
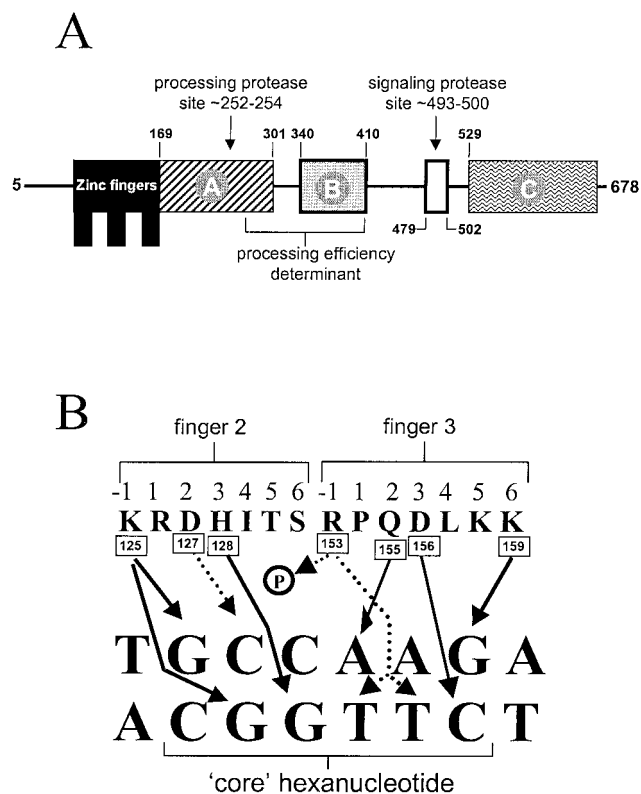
FIG. 2. Schematic representation of PacC and its DNA binding specificity. (A) Functionally relevant regions in PacC are shown, with limits indicated by residue numbers. Interacting regions A, B, and C are required for maintaining the closed PacC conformation (41). The open box denotes the 24-residue signaling protease box (33). The approximate positions of the signaling protease and the processing protease cleavage sites are indicated by arrows. (B) Prediction of specific contacts between residues in the reading α-helix of PacC zinc fingers 2 and 3 and the PacC target hexanucleotide. Experimental evidence and modeling (43) strongly suggest the contacts indicated by the solid lines. Almost every base in both strands of the target site is predicted to establish specific contacts with PacC zinc finger residues. Dotted arrows indicate possible contacts of Asp127 and of Arg153, whose side chain can be modeled as contacting either the phosphate backbone or the O-4 atoms of both the T4′ and T5′ thymines. Note that finger 1 does not appear to be involved in specific contacts with DNA.

The model shown in Fig. 1 accommodates the formal genetic data: at alkaline ambient pH, but not at acidic ambient pH, the products of the six *pal* genes transduce a signal able to trigger the conversion of the transcription factor encoded by *pacC* to an active form able to activate the expression of alkali-expressed genes and to prevent the expression of acid-expressed genes.

PacC Transcription Factor

The derived sequence of PacC contains 678 amino acids (122). An M5I mutation (*pacC504*) (122) has been used to show that most, possibly all, translation proceeds from methionine codon 5, which would result in a 674-residue protein (83). (Residue numbering, however, continues to be on the basis of 678 residues to avoid confusion between earlier and later publications [83].) The most notable feature of the de-

rived translation product is the presence of three Cys_2His_2 zinc fingers beginning at residue 78 (Fig. 2A). A number of regions are rich in certain amino acids (95), but the significance of this is unclear with the possible exception of a glycine-plus-proline-rich region beginning at residue 314 (see below) (41, 122). An eye-catching feature is the presence of three perfect copies and one imperfect copy of a 6-residue direct repeat in which the perfect copies contain only acidic and glutamine residues (122). Its significance is unclear but is not likely to be great, since it is not present in isofunctional homologues from organisms as closely related as *Aspergillus niger* and *Penicillium chrysogenum* (76, 119).

The specificity of the PacC zinc finger DNA binding domain has been thoroughly analyzed using site-directed and classical mutagenesis of both the DNA target and finger residues involved in critical contacts, various footprinting techniques, quantitative binding experiments, and molecular modeling (43, 122). These analyses indicate that finger 1 interacts with finger 2 rather than directly with the DNA and that finger 2 participates in extensive contacts with the 5′ moiety of the binding site while finger 3 binds the 3′ moiety (43). Both partially purified PacC obtained from mycelial extracts and bacterially expressed PacC bind DNA specifically, the core target sequence being 5′-GCCARG with a preference for T at −1 (43, 95, 122). Nearly every base of the core binding sequence appears to be involved in specific contacts (Fig. 2B) (43). A Gln155-to-A4 contact (Fig. 2B) is crucial for binding, which is abolished by either a Gln155Lys substitution or an A4-to-T transversion (43, 95). However, although the sequence specificity for a single PacC binding site is very high, a tandem arrangement of two sites in inverse orientation allows a relaxation of specificity (39). The almost complete conservation of amino acid sequence in the reading (specificity determining) α-helices of fingers 2 and 3 between PacC and its yeast homologues (see below) strongly suggests that these factors will all show the same binding specificity. Indeed, a functional Yl (*Yarrowia lipolytica*) Rim101p site has been shown to be a direct decameric repeat containing two direct repeats of the above consensus (7).

Activation and Repression Roles of PacC

Detailed studies have been made of the roles of PacC in the promoters of one alkali-expressed gene, *ipnA*, and one acid-expressed gene, *gabA* (39, 40).

The bidirectional promoter lying between the divergently transcribed *acvA* and *ipnA* genes contains five GCCARG sites (designated ipnA1 through ipnA4B) within the 872 bp separating the coding regions of the two genes (40). The site closest to *acvA* (ipnA1) has a negligible affinity for a bacterially expressed PacC fusion protein (40). The two sites closest to *ipnA* are in inverse orientation, separated by 9 bp, and form the double ipnA4AB site starting at −258 relative to the major transcription start point (−699 with respect to the translation start). The ipnA2 and ipnA3 sites are located at −593 and −502 relative to the transcription start point, respectively. Interestingly, although ipnA2 has a fivefold-greater affinity for PacC than does ipnA3 or the double ipnA4AB site, deletion of both ipnA3 and ipnA4AB almost completely abolished the elevation of *ipnA* expression under alkaline growth conditions

as monitored by a fusion reporter gene (40). Deletion of ipnA2 alone reduced expression by nearly half, and triple deletion of ipnA2, ipnA3, and ipnA4AB reduced expression about 20-fold, making the expression level not significantly different from that seen under acidic growth conditions (40). Deletion of ipnA3 reduced expression fivefold (40), and it has also been suggested that ipnA3 is the most important PacC binding site for *acvA* expression (121). The fact that deletion of PacC binding sites can eliminate the elevation of *ipnA* expression which occurs under alkaline growth conditions confirms the direct action of PacC in activating the expression of an alkali-expressed gene (Fig. 1).

The *gabA* promoter contains two inversely oriented, adjacent, PacC binding sites, one a consensus GCCAAG site and the other a near-consensus GCCGAG site (39). These sites overlap the site for the transcription factor IntA/AmdR mediating induction by ω-amino acids, with the consequence that PacC and IntA/AmdR compete for binding (39). Thus, PacC repression of *gabA* expression is direct and occurs through blocking induction. Exchanging IntA/AmdR binding sites between the *gabA* and *amdS* (encoding acetamidase) genes renders *gabA* expression independent of ambient pH and *amdS* an acid-expressed gene (39).

Mutational Analysis of *pacC*

pacC has been thoroughly analyzed genetically, and the molecular characterization of *pacC* mutations (33, 41, 43, 82, 95, 122) has been crucial for the understanding of PacC activation by proteolysis (see below). Mutational truncations removing between 92 and 412 residues from the C terminus of PacC result in an alkalinity-mimicking (*pacC*^{c}) phenotype (83, 95, 122; Rainbow et al., unpublished), which revealed that the C-terminal region of PacC contains a negative-acting domain that is inactivated in the presence of alkaline-ambient-pH signaling. Single-residue changes involving residues 259, 266, 340, 573, and 579 also lead to an alkalinity-mimicking *pacC*^{c} phenotype (41, 83). The relatively large size of the region in which alkalinity-mimicking mutations can occur accounts for the ease with which they can be selected and for the erroneous proposal (17), based on their high frequency, that they constitute the loss-of-function class.

Many more N-terminal truncations result in an acidity-mimicking phenotype (partial loss-of-function *pacC*$^{+/-}$ mutations), and if the truncation extends into the zinc finger domain, a null *pacC*$^-$ phenotype results (83, 122; C. V. Brown, L. A. Rainbow, J. Tilburn, and H. N. Arst, Jr., unpublished data). The null phenotype of *pacC*$^-$ mutations includes cryosensitivity of growth at the permissive pH of 6.5, a low growth rate at the permissive temperature, poor conidiation, and overproduction of a brown, presumably melanin, pigment (122; Brown et al., unpublished). Single-residue changes within the DNA binding domain result in an acidity-mimicking phenotype whose severity correlates with the relative importance of the residue in question in DNA binding (43; Brown et al., unpublished). Truncations between residues 299 and 315 also result in an acidity-mimicking phenotype, as do a truncation at residue 379 and a deletion of residues 465 through 540 (41, 83, 122; Brown et al., unpublished). Some explanation for this is given in the sections on processing and interactions (see below), but it

should also be remembered that anything which reduces the amount of PacC, such as *pacC* mRNA or PacC instability, will make the phenotype more acidity mimicking to an extent commensurate with the reduction in PacC levels.

Neutrality-mimicking *pacC*$^{c/-}$ mutations tend to occur in the relatively short region between alkalinity-mimicking and acidity-mimicking truncating mutations (83; Brown et al., unpublished).

Proteolytic Processing of PacC

The analysis of wild-type and mutant forms of PacC in protein extracts prepared from mycelia grown at different ambient pHs provided an explanation for the gain-of-function phenotype of mutations truncating the C-terminal region of PacC. Electrophoretic mobility shift assay (EMSA) and Western analysis revealed two different forms of PacC, the 72-kDa translation product and a 27-kDa truncated version (encoded by codons 5 through ~252 to 254) (83, 95), denoted the processed form. The translation product predominates under acidic growth conditions or in the absence of a functional *pal* pH signaling pathway, whereas the processed form predominates under alkaline growth conditions or in alkalinity-mimicking *pacC*^{c} mutants, supporting the conclusion that PacC is proteolytically processed in response to alkaline ambient pH (41, 83, 95). In agreement, the translation product and the processed form show a precursor-to-product relationship in a *pal* pathway-dependent manner (83). As noted above, the major class of mutant *pacC*^{c} alleles leading to an alkalinity-mimicking phenotype results from C-terminal truncation of PacC. Mutant proteins truncated at or between residues 407 and 578 are proteolytically processed at any ambient pH, independently of the *pal* pathway, indicating that a key function of the C-terminal moiety removed by *pacC*^{c} truncating mutations is prevention of proteolytic processing under acidic ambient pH conditions.

What is the physiological role of PacC processing? The translation product is relatively inert transcriptionally, as shown by the strong acidity-mimicking phenotype resulting from either nonleaky *pal*$^-$ mutations, preventing proteolytic processing (31, 32, 91, 95) and leading to preferential cytosolic localization of PacC (82), or nontruncating *pacC*$^{+/-}$ mutations (e.g., the *pacC*$^{+/-}$20205 deletion and the *pacC*$^{+/-}$209 missense mutation [33, 83]), which result in mutants that are phenotypically indistinguishable from null *pal* mutants. In contrast, a mutant PacC protein truncated after residue 266 (the *pacC*^{c}50 product) is able both to activate "alkaline" gene and to repress "acidic" gene expression (95). Since the C terminus of this mutant protein is close to that of the processed form(s) (within residues 252 to 254), this finding indicates that processing leads to the activation of the otherwise inactive *pacC* translation product. In view of the facts that PacC[5–250], PacC[5–265], and PacC[5–273] polypeptides tagged at their C termini (in all three cases close to the processing site) with green fluorescent protein (GFP) are localized in the nucleus in a *pal*-independent manner (81, 82) and that PacC[5–250] tagged at its N terminus with GFP is also localized in the nucleus in a pH- and *pal*-independent manner (81, 82), PacC proteolytic activation clearly at least promotes the nuclear localization of a transcriptionally active polypeptide. The model accommodating this

finding in references 82 and 95 has been modified to take into account the fact that the proteolytic activation of PacC takes place in two steps (see below and Fig. 4).

Closed and Open Forms of PacC

The products of *pacC^c* alleles are processed at acidic ambient pH, as is the largely nonfunctional but processable *pacC^{+/−}*230 product (83, 95) (see below), which strongly suggests that the processing protease is not pH (or PacC) regulated. Therefore, the susceptibility of PacC to the processing protease appears to be the pH-regulated, *pal* signal requiring step.

The existence of two different forms of the translation product was proposed to accommodate both the pH independence of processing and the opposing phenotypes of two mutations which have been crucial for understanding the proteolytic processing cascade (83): *pacC^c*69(L340S) is a missense mutation (33, 83), leading to alkalinity mimicry. PacC^{L340S} is processed irrespective of ambient pH (33, 83), suggesting that its translation product is in an "open" conformation, permanently accessible to the protein protease even in the absence of *pal* signaling, and that Leu340 is essential for maintaining a protease-inaccessible "closed" conformation, preventing PacC proteolytic activation in the absence of *pal* signaling (i.e., under inappropriate circumstances). *pacC^{+/−}*20205 is a strong loss-of-function mutation phenotypically indistinguishable from a *pal^−* mutation (83). The *pacC^{+/−}*20205 product is largely unprocessed at both acidic and neutral (where pH signaling occurs; note that *pacC^{+/−}* mutations prevent growth at alkaline pH [Table 1]) pHs (41, 83). This mutant PacC, with residues 465 to 540 deleted, is deficient in pH signal reception and response and appears to be locked in the closed conformation.

That in vitro-synthesized PacC^{L340S} was more susceptible to a proteolysis by a mycelial extract than was the wild-type protein (41) strongly supports the view that the Leu340Ser substitution causes a conformational change in PacC. Moreover, a classically selected mutation corroborates the view that the opposing phenotypes of *pacC^c*69(L340S) and *pacC^{+/−}*20205 result from opposing effects of the mutational changes on PacC accessibility to the processing protease. *pacC^c*2020507 is an intragenic suppressor of *pacC^{+/−}*20205 leading to alkalinity mimicry and processing of the doubly mutant PacC at any ambient pH. It results in a Leu340Ser substitution (41), which demonstrates that the processing defect of *pacC^{+/−}*20205 results from inability to respond to ambient pH by adopting the open conformation.

The molecular characterization of *pacC^c* truncating mutations leading to pH-independent processing strongly implicated a C-terminal region of PacC in maintaining the protease-inaccessible conformation. In agreement, one- and two-hybrid, coimmunoprecipitation and affinity column experiments showed that a polypeptide containing residues 529 to 678 was able to interact with upstream regions contained within residues 169 to 410 in a Leu340-dependent manner, demonstrating that this interaction corresponds to that disrupted by L340S (*pacC^c*69) and is therefore physiologically relevant (41). A key finding was that interactions involving PacC polypeptides are strong enough to be detectable by a modified EMSA (41). A truncated PacC form (and therefore open) DNA complex was
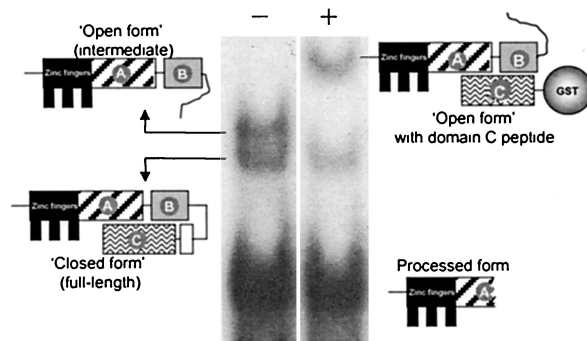

FIG. 3. Supershift assay. The full-length, intermediate, and processed PacC-DNA complexes can be resolved in an EMSA gel. The open (intermediate) form has its regions A and B available for interaction with a purified glutathione *S*-transferase (GST) fusion protein (here GST::PacC[410–678]) containing interacting region C, whereas in full-length, closed PacC these regions are not available (see the schematic representations). The remarkably stable interaction of open PacC with the large (possibly dimeric) GST fusion protein results in markedly reduced mobility (i.e., supershift) of the corresponding protein-DNA complex. The presence or absence from the reaction mixture of the GST::PacC[410–678] fusion protein is indicated by + and −, respectively.

supershifted by the presence in the reaction mixture of a polypeptide containing PacC residues 529 to 678, expressed, and purified from recombinant bacteria (Fig. 3). The relative ease with which different PacC regions could be monitored in this assay facilitated the identification of three interacting regions in PacC, denoted A, B, and C (Fig. 2A) and contained within residues 169 to 301, 334 to 410, and 529 to 678, respectively (41). Interacting regions A and B include critical determinants for the efficiency of the processing protease (33; E. Díez and M. A. Peñalva, unpublished data), and region A overlaps the processing site, which strongly suggests that access of the processing protease is sterically prevented in the closed conformation (41).

In *A. nidulans* PacC and its most closely related homologues (e.g., in *A. niger* and *P. chrysogenum*), regions A and B are separated by a Pro- and Gly-rich linker (119), which is proposed to serve as a hinge enabling opening from the closed conformation in response to alkaline ambient pH signaling (41). The interaction of regions A and B would be held in place by region C, which explains why C-terminal truncating mutations removing this domain result in pH-independent processing and alkalinity mimicry.

Single-residue substitutions disrupting the closed conformation were obtained by classical genetics in each of the interacting domains, bypassing the need for pH signal transduction. These included Leu259Arg (*pacC^c*63) and Leu266Phe (*pacC^c*39) in region A, Leu340Ser (*pacC^c*69) and the phenotypically weaker Leu340Phe (*pacC^c*234) in region B, and Arg573Trp (*pacC^c*2020510), Arg579Gly (*pacC^c*232), and Arg579Thr (*pacC^c*200 *pacC*20042) in interacting domain C (41, 83)

The supershift assay provided further evidence for disruption of the interactions involving interacting regions A, B, and C by the Leu340Ser substitution. In wild-type PacC obtained using acidic growth conditions, region C, which is involved in intramolecular interactions with upstream regions, could not

participate in intermolecular interaction with a purified poly-peptide from residues 169 to 410 containing A and B, whereas it could participate in this interaction in the L340S mutant (41). The same situation was found with the L259R mutant, confirming the similarity of consequences of substitutions affecting crucial residues in either A or B (41).

Physiological Relevance of the Open Form

The supershift assay provided a biochemical way of defining the open PacC form as the proportion of unprocessed PacC whose interacting regions A and B are available for interactions with a domain C (residues 529 to 678) polypeptide (41). Mutant proteins lacking all or a significant portion of this domain are in the open conformation, and, if truncation occurs downstream of residue 407 (the downstream limit of a processing efficiency determinant) (83), they are also committed to processing (33).

If the closed-to-open conformational transition is the pH-regulated step, the relative proportion of each form should vary according to ambient pH in a *pal*-dependent manner. Espeso et al. (41) demonstrated that the relative proportion of open to closed form in unprocessed PacC is twice as great at alkaline as at acidic pH. This response to ambient pH was largely missing in a *palA*1 mutant strain. Amounts of the open form were much reduced in the *palA*1 strain and even more so in a *pacC*$^{+/-}$20205 strain (41).

Processing Protease Step

The processing protease step leads to the PacC processed form, which appears to be slightly heterogeneous in size. Processing does not remove residues from the N terminus of PacC (83; E. Díez, M. A. Peñalva, and J. Tilburn, unpublished data), enabling high-resolution (~500-Da discrimination) EMSA gels to be used to determine that the C-terminal residue of the most upstream processed form lies within residues ~252 to 254 (~27 kDa) (83). An unexpected feature of the processing proteolysis was revealed by the fact that mutant proteins having deletions of residues 235 through 264 or 235 through 301, which remove the processing protease target site(s), are processed to polypeptides whose size is indistinguishable by EMSA from that of the wild type (83; Díez and Peñalva, unpublished). This strongly suggests that the specificity of the processing protease resides at sequence or structural determinants located upstream of residue 235 and that the protease cuts at peptide bonds remote from these determinants, thereby resembling type 1 endonucleases (83). Identification of this remarkable processing protease has thus far proved elusive. The *palB* calpain-like product is not the processing protease (32) (see below), and no other Pal protein has sequence signatures characteristic of known proteases. *pal*$^-$ mutations are always hypostatic to *pacC*^{c} mutations even though the PacC proteins of *pacC*^{c} strains undergo processing (see above). Thus, the gene(s) encoding the processing protease remains to be identified. It should be noted that the processing protease appears to be conserved in *Saccharomyces* (83), although it does not appear to recognize the isofunctional PacC homologue Rim101p (135) (see the *Saccharomyces* discussion below).

In addition to specificity determinants upstream of residue 235, the efficiency of the processing protease is largely dependent on residues 266 to 407 (33, 83; Díez and Peñalva, unpublished). Impaired processing of mutant proteins lacking this efficiency determinant (Fig. 2A), which is dispensable for the signaling protease (see below), revealed the existence of the processing intermediate (33).

Transition from the Closed to the Open Conformation Involves Another Proteolytic Step, the Signaling Proteolysis

High-resolution EMSA (33, 82) discriminates between the open and closed PacC-DNA complexes. PacC in the open complex is not recognized by anti-PacC[529–678] antiserum or, in the case of C-terminally tagged PacC, by antisera against the cognate tag (33), strongly suggesting that open PacC is not the full-length translation product and actually lacks C-terminal residues. Therefore, the closed-to-open transition involves a proteolytic step distinct from the processing step. Two experimental approaches were crucial to demonstrating that this proteolysis, denoted the signaling protease step (33), is the pH-regulated step that is dependent on the *pal* signaling pathway. First, PacC deletion mutants partially or fully deficient in the processing protease step accumulated a processing intermediate truncated between residues ~489 and 511 (i.e., lacking the C-terminal interacting region C) at the expense of the translation product and in a *pal*-dependent manner (33). Second, analysis of PacC in cultures in which the pH was shifted from acidic to alkaline conditions revealed that closed PacC is rapidly converted to open PacC after the pH shift and that this correlates strictly with the detection in Western blots of an intermediate truncated between residues ~493 and 500. As in deletion mutants, this intermediate accumulated at the expense of the translation product in a *pal*-dependent manner (33). This intermediate is the precursor of the processed form (truncated at residues ~252 to 254) (33), as predicted by commitment to processing of similar mutant proteins such as the *pacC*^{c}14 product (truncated after residue 492) (95).

Díez et al. (33) identified a 24-residue conserved sequence, denoted the signaling protease box, which overlapped the predicted C terminus of the intermediate and whose deletion prevented both the signaling and the processing proteolytic steps, in agreement with a translation-product-to-intermediate-to-processed-form proteolytic cascade (Fig. 4). Two classically selected mutations in which Leu498 located within the signaling protease box was replaced also fully (Leu498Ser) or partially (Leu498Phe) prevented the formation of both the intermediate and the processed form (33), revealing a crucial role for Leu498 in the formation of the intermediate. *pacC*$^{+/-}$209, the strongest of the above mutations, is an extreme-acidity-mimicking mutation, phenotypically indistinguishable from null alleles in any of the *pal* genes except *palI* (see above) (33). The requirement of the signaling protease box for the formation of the intermediate and processed forms explains why the *pacC*$^{+/-}$20205 product is locked in the closed conformation (see above), since the *pacC*$^{+/-}$20205 deletion removes this conserved 24-residue sequence (Fig. 2A) (33).

The extreme-acidity-mimicking *pacC*$^{+/-}$209 mutation and the molecular nature of the blocked reaction (an irreversible proteolytic cleavage of PacC) strongly suggest that this is the
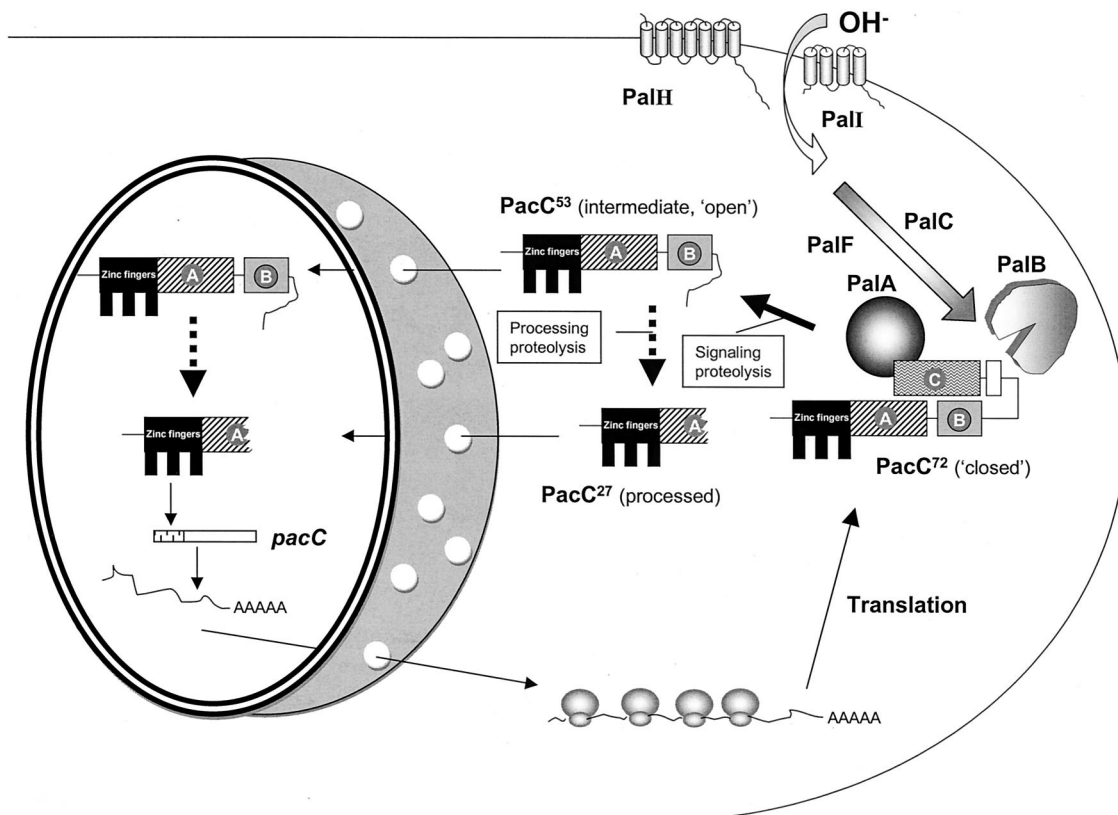
FIG. 4. Current two-step model of PacC proteolytic activation. Details are described in the text. PacC[72], PacC[53], and PacC[27] refer to the 72-kDa full-length, the 53-kDa intermediate, and the 27-kDa processed PacC forms, respectively. The signaling cleavage step requires alkaline pH signaling through the *pal* pathway and leads to the intermediate, which is committed to the ambient pH-independent processing cleavage step. The processing protease has not yet been identified, but PalB would appear to be the signaling protease recognizing the signaling protease box (shown as an open box). Commitment to processing results from removal of interacting region C, which is also removed by *pacC^c*-truncating mutations. That the signaling cleavage occurs in the cytosol is supported by the nuclear exclusion of its PacC[72] substrate and the preferential nuclear localization of its PacC[53] product (82). The processing cleavage might be in the cytosol and/or in the nucleus, as indicated by the dotted lines connecting PacC[53] and PacC[27]. *pacC* is itself an alkali-expressed gene (122).

ultimate event in signaling by the *pal* pathway. The only protease thus far identified in this pathway is the calpain-like PalB protein (see below), implicating it as the likely candidate for the signaling protease (33). In this context, the fact that that calpain, which shows no strong sequence specificity for cleavage, nevertheless shows some preference for Leu in position P2 (21) is very suggestive in view of the critical role shown for Leu498. However, definitive evidence confirming the role of PalB as the signaling protease remains to be provided.

Díez et al. (33) also showed that PacC[L340S], in which interactions between regions A, B, and C have been disrupted by the Leu340 substitution (see above), undergoes processing (abnormally) without passing through the intermediate, which in turn provides further support for the conclusion that the key role of the signaling cleavage lies in disrupting the interactions which prevent processing under inappropriate circumstances.

Figure 4 summarizes the current two-step model for the proteolytic activation of PacC (33). The *pal*-regulated signaling protease cleavage takes place within the signaling protease box, leading to the intermediate which was formerly denoted "open PacC" (41, 83). This intermediate lacks interacting re-

gion C and is therefore committed to processing. Readers should note that this two-step processing of PacC can be compared to Rip (regulated intramembrane proteolysis) (10, 11).

Regulation of PacC Nuclear Localization

Ambient-pH-dependent proteolytic activation regulates PacC subcellular localization (82). In the absence of pH signaling, the translation product is largely cytosolic. In contrast, its conversion to the processed form resulting from pH signal reception correlates with almost exclusive nuclear PacC localization. The *palA*1 mutation, which prevents processing at neutral pH, also prevents this transition from cytosolic to nuclear localization (82). The open PacC polypeptide (i.e., the intermediate truncated after residue ~500) also appears to be nuclear (82). Therefore, formally, the C-terminal moiety removed by processing prevents the nuclear localization of PacC (82). The mode by which the C-terminal region of PacC prevents its nuclear localization is unknown. Figure 4 incorporates data on the different subcellular distributions of the PacC translation product and the intermediate and processed forms.

PacC contains a putative bipartite nuclear localization signal

TABLE 2. Conservation of the *pal* signaling pathway

A. nidulans	S. cerevisiae	Function	Mammals
PalA	Rim20p	Interacts with Rim101p/PacC	Alix/AIP1 (ALG2-interacting protein)
PalB	Rim13p	Calpain-like cysteine protease	*CAPN7* calpain
PalC	None	Unknown	
PalF	Rim8p	Unknown	
PalH	Rim21p	7 TM membrane protein	GPCRs ?
PalI	Rim9p	4 TM membrane protein	

between residues 252 and 269 (122), which is insufficient to result in nuclear localization of a GFP reporter (82). This sequence might facilitate nuclear localization of the processing intermediate but not of the processed form, since it is removed by the final processing step. Therefore, a nuclear localization signal located within residues 5 through ~252 to 254 must mediate the *pal*-independent nuclear localization of the GFP-tagged processed form (82). Using deletion analysis, Mingot et al. (82) showed that within these limits only the zinc finger region (residues 66 through 173) mediates nuclear localization of GFP. The possibility that the 53.8-kDa GFP-tagged processed form is localized in the nucleus following passive diffusion and nuclear retention by DNA binding, rather than by active nuclear localization signal-mediated nuclear import, was discarded for the following reasons. (i) In gel filtration experiments, this GFP-tagged protein elutes as a 101-kDa protein, which would be above the exclusion limit of nuclear pores. (ii) PacC residues 5 to 234 (including the zinc finger region) drive the nuclear localization of *Escherichia coli* β-galactosidase. (iii) the Gln155Lys substitution involving a residue in the third PacC finger prevents DNA binding (see above) but not nuclear localization. Therefore, the PacC zinc finger region not only is involved in DNA binding but also contains a functional nuclear localization signal (82).

pH Signal Transduction Pathway

All six *pal* genes of the pH signal transduction pathway have been characterized (3, 31, 32, 77, 90, 91). With the exception of PalB, inspection of the amino acid sequences of their products gives very few clues to molecular functions, strongly suggesting that the *pal* signaling pathway is mechanistically novel (Table 2).

Two of the conceptually translated proteins contain putative transmembrane (TM) domains. PalI, with 549 residues, contains four putative TM domains within the N-terminal one-third of the protein (31). The C-terminal two-thirds of the protein is hydrophilic. The Gly47Asp substitution in the predicted acidic loop between transmembrane segments 1 and 2, in a region highly conserved with the *Saccharomyces* isofunctional homologue, abolishes PalI function (31). It has been suggested that PalI might be a membrane sensor for ambient pH and that this loop, predicted to be periplasmic (i.e., between the plasma membrane and cell wall), might play an important role (31). The null phenotype for *palI* mutations is less extreme than the null phenotype for the other five *pal* genes of the pH signaling pathway (3, 31, 32, 90, 91, 122), indicating that some signal can be transduced in the absence of

PalI. Therefore, either PalI is not the only pH sensor or it functions in sensing ambient pH in cooperation with other proteins with which its participation is not essential.

A second Pal protein shares with PalI a predicted membrane localization. The derived PalH sequence contains 760 residues with seven putative TM domains in the N-terminal moiety followed by a hydrophilic C-terminal moiety of just over 400 residues (31). As in seven TM plasma membrane receptors, this long, hydrophilic C-terminal moiety is predicted to be cytosolic. Interestingly, it is not absolutely essential for pH signal transduction, since *palH* frameshift mutations truncating the normal sequence after residues 377 and 598 have a leaky, partial-loss-of-function phenotype similar to that of a null *palI* mutation (91). Double mutants carrying a leaky *palH* mutation and a *palI* mutation have a phenotype indistinguishable from those of null mutations in *palA, palB, palC, palF,* or *palH,* indicating additivity (91). The possibility that PalH is a seven-TM-domain plasma membrane receptor seems rather attractive in view of the possible involvement of endocytosis in pH sensing, to be described in the *Yarrowia* and *Saccharomyces* sections (see below).

Conceptual translation of the *palB* sequence yielded an 847-residue polypeptide having convincing similarity to the catalytic domain in the large subunit of calpains (Ca^{2+}-dependent cysteine proteases) (32). Prototypical mammalian m-calpain consists of large and small subunits, both containing calmodulin-like penta-EF-hand (5-EF-hand) motifs which account for Ca^{2+} binding and dependence (for reviews, see references 113 and 114). PalB, containing the calpain catalytic triad consisting of Cys (providing the active site thiol), His, and Asn residues (32), is an atypical calpain in that it lacks the large calpain subunit 5-EF-hand domain (32). Extrapolation from work with *Saccharomyces cerevisiae* (see below) indicates that the presumably catalytic Cys residue, completely conserved among PalB homologues, is essential for PalB function (48).

PalB cannot be responsible for the final proteolytic processing of PacC because a *palB* allele with a null phenotype fails to prevent pH-independent processing of a mutant PacC lacking 214 C-terminal residues (i.e., lacking interacting region C) to yield a processed form indistinguishable from that of the wild type (32). PalB is therefore a likely candidate for the signaling protease removing PacC interacting region C, converting the PacC translation product into the processing-committed intermediate (33). This would imply that the highly conserved signaling protease box (Fig. 2A) is likely to be the PalB recognition and cleavage site (33).

Using amino acid sequence alignments, Sorimachi et al. (113) identified in fungal PalB homologues a highly conserved region located downstream of the putative PalB catalytic domain, which they designated PHB (for "PalB homology Domain"). This region is diagnostic of a subfamily of calpains which includes metazoan homologues, such as *Caenorhabditis elegans* CE01070, another atypical calpain lacking the 5-EF-hand domain (113, 114). This raises the intriguing possibility that homologues of fungal ambient pH transducers are conserved in metazoans. Indeed, Futai et al. (47) reported the characterization of a human gene, *CAPN7*, encoding PalBH/Capn7, a human homologue of PalB of unknown function, which shows preferential nuclear localization. This finding un-

derscores the utility of fungal pH signal transduction genes to reveal unexpected gene features in metazoans.

The 798-residue derived PalA sequence provides few clues to its molecular role (90). The presence of several SH3 domain binding motifs and a proline-rich region (90) possibly hint at protein-protein interactions. Xu and Mitchell (135) have shown that the *S. cerevisiae* PalA homologue interacts with the *S. cerevisiae* PacC homologue and suggested that such an interaction might be important for the action of the *S. cerevisiae* PalB homologue (see below). They have shown that the interaction is conserved in *Candida albicans*. It is also conserved in *A. nidulans* (O. Vincent and M. A. Peñalva, unpublished data), lending further support to the notion of its generality. The possible relationship of PalA and its fungal homologues with endocytosis is described in the *Saccharomyces* and *Yarrowia* sections (see below).

As in the case of PalB, homologues of PalA are present in the animal kingdom, and their molecular characterization strongly suggests that a PalA-like domain is involved in signal transduction pathways in eukaryotes from fungi to mammals. Murine Alix/AIP1 is a PalA homologue which interacts with ALG-2, a protein required for apoptosis triggered by a number of stimuli and a member of the calpain small-subunit subfamily of calcium binding proteins (66, 74, 84, 130). Overexpression of a truncated form of Alix/AIP1 lacking the N-terminal moiety can protect against cell death (130). Overexpression of the human PalA and Alix/AIP1 homologue HP95 inhibits the growth of confluent HeLa cells through G_1 arrest (133a). Another PalA homologue is the rat protein p164$^{PTP-TD14}$, which can inhibit Ha-*ras*-mediated transformation (20). The C-terminal moiety of p164$^{PTP-TD14}$ contains a putative protein tyrosine phosphatase that is apparently required for inhibition of Ha-*ras*-mediated transformation, whereas the N-terminal moiety contains the region of similarity to PalA (20). Other PalA homologues are Xp95, suggested to be an element in a tyrosine kinase signaling pathway involved in progesterone-induced *Xenopus* oocyte maturation (23), and YNK1, a *Caenorhabditis elegans* protein of unknown function (24).

The 775- and 507-residue derived PalF and PalC sequences (77, 91), respectively, provide no clues to their functions. A leaky, partial-loss-of-function *palF* mutation probably gives no information about a single residue or region since it is a frameshift which would exchange the C-terminal 6 residues of PalF, conserved in closer but not more distant homologues, for a highly hydrophobic 56-residue peptide which presumably adversely affects PalF function, stability, solubility, or subcellular localization (H. J. Bussink and H. N. Arst, Jr., unpublished data). This partial-loss-of-function *palF* mutation is additive in phenotypes with *palI* mutations and partial-loss-of-function *palH* mutations, with the double mutants having the stringent null phenotype of *palA, palB, palC, palF*, and *palH* mutants (91). The most interesting point that can be made at present about *palC* is the absence of an identifiable homologue in the *S. cerevisiae* genome (91), although one is present in *Neurospora crassa*. The distribution of *palC* homologues within the fungal kingdom is therefore likely to be of interest. The (extremely limited) current evidence suggests that *palC* might be present only in filamentous fungi (see below).

Loss-of-function mutations in any of the six *pal* genes prevent PacC processing (31, 32, 82, 83, 91, 95) because they prevent the formation of the intermediate, the processing protease substrate (33). This feature is less extreme in *palI* and leaky *palF* and *palH* mutants (31, 91). (Leaky mutations in *palA, palB*, and *palC* have yet to be obtained.) However, additivity of mutations causing less extreme phenotypes can be seen in EMSA, as can the epistasis of a nonleaky, stringent *pal* mutation to a *palI* mutation (31, 91). Alkalinity-mimicking *pacC^c* mutations are epistatic to acidity-mimicking *pal* mutations, and this epistasis is seen both when monitoring in vivo characteristics (3, 17) and when using EMSA or Western blot analysis to determine the amount and extent of processing of PacC (95).

Presence of the pH Regulatory System Homologues in Other Fungi

As noted in several sections of this review, homologues of *A. nidulans pacC* have been identified in a number of filamentous ascomycetes, including *A. niger* (76), *Penicillium chrysogenum* (119), *Acremonium chrysogenum* (110), *Sclerotinia sclerotiorum* (106), and *Aspergillus oryzae* (M. Sano and M. Machida, GenBank accession no. AB035899). Therefore, it will come as no surprise to readers that other filamentous ascomycetes such as *Aspergillus fumigatus* (The Institute for Genomic Research [http://www.tigr.org]) and *Neurospora crassa* (Whitehead Institute/MIT Center for Research [http://www-genome.wi.mit.edu]) have homologues of all the identified genes involved in pH regulation in *A. nidulans*, including *palC*. As described in detail in the following sections, the budding yeast *Saccharomyces cerevisiae* and two dimorphic yeasts, *Candida albicans* and *Yarrowia lipolytica*, also have homologues of the pH regulatory system genes, with the sole exception of *palC*. Therefore, the pH regulatory system appears to be universally present in all major groups of ascomycetes: hemiascomycetes, plectomycetes, and pyrenomycetes.

pH REGULATION IN *SACCHAROMYCES CEREVISIAE*

Molecular Characterization of the *RIM* Regulatory System

RIM101, formerly *RIM1* (*rim* stands for "regulator of *IME2*" [see below]), encodes the founding member of the RIM101p/PacC group of transcription factors. RIM101p is a positive regulator of meiosis in budding yeast, through its stimulation of transcription of *IME1*, a gene encoding a key positive-acting transcription factor which activates a number of meiotic genes, including the kinase-encoding gene *IME2* (117, 118). Nonallelic, recessive *rim101, rim8, rim9*, and *rim13* mutations result in a similar phenotype, characterized by reduced expression of *ime1::HIS3* and *ime2::lacZ* gene fusions, cryosensitive growth, and smooth colony morphology (117, 118). As noted above (Table 2) and below, *RIM8, RIM9*, and *RIM13* are the budding yeast homologues of *A. nidulans palF, palI*, and *palB*, respectively.

RIM101 was cloned as restoring *ime2::lacZ* expression in a *rim101-1* mutant, taking advantage of its close linkage to *RIM4*, a gene in which inactivating mutations completely prevent sporulation. This facilitated the isolation by complementation of plasmids covering the *RIM101-RIM4* genomic region (118). A *rim101Δ* mutant was phenotypically indistinguishable from

classical *rim101, rim8, rim9*, and *rim13* mutants, in agreement with the conclusion that recessive *rim101* classical mutations represent the loss-of-function class. Rim101p is a 628-residue polypeptide (118) (note that the sequence reported in Gen-Bank P33400 by the yeast-sequencing group contains only 625 residues) having three zinc fingers essential for function (118). Despite the similar sizes of PacC and Rim101p, significant amino acid identity is almost completely restricted to the zinc finger region.

In addition to the above phenotypic features, *rim101, rim8, rim9, rim13*, and *rim20* mutations prevent haploid invasive growth (71, 135). This invasive growth resembles pseudofilamentation in yeast diploids and is defined as a combination of filament formation (which implies a switch from axial to polar budding mode) and penetration of agar underneath colonies growing on rich medium (105). Like diploid pseudohyphal growth, invasive growth requires the mitogen-activated protein kinase cascade components Ste20p, Ste11p, Ste7p, and Ste12p involved in the mating pheromone response pathway. It can also be induced independently of the mating pheromone pathway, for example through the Tpk2p cyclic AMP-dependent-protein kinase A pathway (for reviews of *Saccharomyces* pseudohyphal growth, see references 51 and 70). The functional interaction between the *RIM* pathway and the last two pathways promoting filamentation remains to be addressed. The fact that the *RIM* pathway is important for the transition from the yeast to the hyphal mode of growth in the pathogenic yeast *Candida albicans* is addressed below. Readers should note that Cph1p, the *C. albicans* Ste12p homologue, also plays a role in filamentation (73).

Rim101p Is Activated by Proteolytic Processing, Removing a C-Terminal Region

In common with PacC, Rim101p undergoes proteolytic processing activation which, in this case, removes approximately 100 residues from the C terminus of the protein (71, 135). The relative levels of the Rim101p translation product and the processed form depend on growth medium composition. As with PacC, media containing high glucose levels, whose catabolism leads to external acidification, promote accumulation of the translation product, whereas the processed form largely predominates on alkalinization (to pH 8.0) of the growth medium or in the presence of acetate as a carbon source (71), which leads to a final culture pH close to neutrality, where pH signaling also takes place.

The *rim9, rim8, rim13*, and *rim20* loss-of-function mutations prevent the proteolytic processing activation of Rim101p (68, 71, 135). In agreement with the activating role of Rim101p processing, mutations truncating the protein after residue 485, 531, or 539, which would be formally equivalent to *pacC^c* gain-of-function mutations truncating the C-terminal region of PacC, suppress a *rim9* mutation (71). In contrast, more upstream truncating mutations do not (71). This would suggest that essential Rim101p regions where truncating mutations would be equivalent to the partial loss-of-function *pacC$^{+/-}$* class lie upstream of residue 485. However, this conclusion should be reexamined using site-directed mutagenesis to introduce stop codons as appropriate, rather than using 3′ nested deletions, which might lead to transcript instability, complicat-

ing phenotype interpretation (examples are described below in the *Yarrowia* section). In summary, a C-terminal region in Rim101p downstream of residue 531 behaves as a *pal (RIM)* signaling pathway-dependent negative-acting region. In agreement, an epitope-tagged protein truncated after residue 531 was shown to suppress *rim8, rim9*, and *rim13* mutations by restoring *ime1::HIS3* and *ime2::lacZ* expression and invasiveness and overcoming the cryosensitivity phenotype associated with these mutations (71). In contrast a protein truncated after residue 620, which lacks the eight C-terminal residues, does not suppress a *rim9* mutation, indicating that the negative-acting, *RIM*-dependent region involves more than this C-terminal tip of the protein (assuming that the mutation has not affected mRNA stability).

One-Step Processing

A difference between Rim101p and PacC is that whereas Rim101p proteolytic activation requires one *RIM* pathway-dependent proteolytic step (71, 135), PacC activation occurs in two sequential steps, the first of which (the signaling proteolysis) is pH regulated through the *pal* pathway and the second of which (the processing proteolysis) appears to be pH independent (33) (see above). The position of the single proteolysis at Rim101p was estimated at ~100 residues from C-terminal residue 628 (71, 135). The signaling protease cleavage site in PacC lies between residues ~493 and 500, ~180 residues from the C-terminal residue 678 and markedly downstream of the processing proteolysis site(s) at residues ~252 to 254.

The single *rim* pathway-dependent Rim101p cleavage would be equivalent to the signaling cleavage in the proximity of the C terminus of PacC (33, 135). A plausible candidate enzyme for this *pal (RIM)* pathway-dependent proteolysis is PalB/Rim13p (33, 135), although this reaction has not yet been demonstrated directly. From an evolutionary point of view, it remains to be established whether Rim101p has lost susceptibility to the processing protease or PacC has gained this susceptibility.

Despite the apparent lack of processing-protease susceptibility in Rim101p, the PacC processing protease appears to be conserved in *Saccharomyces*, since PacC[5–492], a mutant protein approximating the PacC intermediate, is processed in yeast (83). In contrast, the yeast *RIM* pathway does not appear to recognize PacC, since processing fails to occur with the full-length wild-type protein (83).

Rim101p and the pH Response

An important question is whether the *RIM* pathway in yeast responds to ambient pH and/or has diverged from the *Aspergillus pal* pathway to respond to a different signal necessary for efficient sporulation in the **a**/α diploid cells and invasiveness in haploids. Several lines of evidence strongly suggest that the *RIM* pathway responds to ambient pH. First, as noted above, Rim101p processing is favored by neutral to alkaline ambient pH conditions and diminishes with acidification of the ambient pH. This would be consistent with inactivation of the *pal (RIM)* signaling pathway, which completely prevents processing, mimicking extremely acidic pHs. It should be noted that in *Aspergillus*, mimicry of acidic pH conditions achieved by mutational

inactivation of the *pal* pathway is more extreme that that usually achieved by chemically manipulating the growth medium pH, particularly when the pH is modified by microbial growth. Therefore, the inability to prevent fully Rim101p processing by lowering the ambient pH might simply reflect an inability to block pH signaling at the acidic pHs in standard use. Second, a previously unmentioned phenotype of *rim101Δ*, *rim20Δ* (68, 135), and *rim13Δ* (48) mutants is that of impaired growth at alkaline pH, which strongly suggests that the *RIM* pathway is required for adaptation to alkaline pH. Third, macroarray transcriptional analysis (see below) revealed that transcription of a subset of genes is elevated by alkaline ambient pH in a Rim101p-dependent manner (68).

Domain of Action of the pH Response

Lamb et al. (68) investigated the genes whose transcription was activated by alkaline pH (8.0) relative to acidic pH (4.0) using macroarray filters containing the yeast open reading frames (ORFs). They identified 71 genes whose transcripts were at least 2.1-fold more abundant in pH 8.0 cells than in pH 4.0 cells. These genes included the Na^+-ATPase structural gene *ENA1*, whose transcription was previously known to be stimulated by both high salt and alkaline stress and which was therefore a positive control. This screen identified, among others, several genes involved either in phosphate or in iron or copper starvation. Comparative Northern and reporter analyses of the relatively high expression of a selection of these "alkaline" genes in *rim101Δ* and *RIM101* cells was used to subdivide them into three different classes, depending on whether their relatively high levels in pH 8.0 medium were (i) strictly dependent on *RIM101*, (ii) partially dependent on *RIM101*, or (iii) independent of *RIM101* (68).

Class i genes include *ARN4* (whose product is involved in iron uptake) and three genes of unknown function. That these genes require the positive action of Rim101p for preferential expression at alkaline pH agrees with the fact that their response to alkaline pH is prevented by a *rim13Δ* allele (68), since an intact *RIM (pal)* pathway is required for the proteolytic activation of Rim101p. As noted above and below, Rim13p is the homologue of PalB, the cysteine protease presumed to remove the processing-preventing interacting domain C in PacC (33). The requirement for Rim13p is bypassed at alkaline pH by the *RIM101-531–HA2* allele (68), which is consistent with the notion that one role of Rim13p involves activation of Rim101p through C-terminal proteolysis of the latter. At pH 4.0, however, transcription of some of the *RIM101*-dependent genes was not increased in the *RIM101-531* mutant. Although this result should be treated with some caution (as noted above, this truncating mutation is a genomic deletion which might cause instability of the incomplete transcript), it might suggest that such genes require, in addition to wide domain regulation provided by active Rim101p, specific induction conditions not provided by the acidic growth medium (a possible example with the *Y. lipolytica AXP1* gene is discussed below).

The fact that class ii genes are partially and class iii genes are fully independent of Rim101p demonstrates that in yeast not all the alkaline response is mediated by this PacC homologue (68). Rim101p-independent class iii genes include *PHO84* (en-

coding a high-affinity phosphate/H^+ symporter) and, paradoxically, *PHO11/12* (encoding phosphate-repressible acid phosphatases). In an independent study, Causton et al. (22) also identified *PHO89* (encoding a phosphate/Na^+ symporter) as a gene whose expression is stimulated by alkaline pH (but the question of Rim101p control was not addressed). All four *PHO* genes listed above are regulated by Pho4p in response to phosphate starvation (94). There is therefore another possible explanation for their apparent classification as alkali-expressed genes, an explanation which might resolve the paradox that genes such as *PHO11, PHO12*, and possibly *PHO84*, whose functions would lead to a prediction of preferential expression under acidic conditions, apparently show preferential alkaline expression. Phosphate-limiting growth conditions were not used in monitoring the expression of these genes (68), but phosphate repression might have been stronger in acidic than in alkaline medium: accumulation of vacuolar polyphosphate is stimulated by acidic growth conditions and dependent on vacuolar acidification mediated by the vacuolar membrane H^+-ATPase (79, 134). Under alkaline growth conditions (leading to vacuole alkalinization through uptake of endocytotic fluid), the predicted decrease in polyphosphate levels might trigger phosphate starvation signaling.

Little is known concerning yeast acidic gene expression. One of the above transcriptional profiling studies (22) identified three genes, *PDR12, ZMS1*, and *TRK2*, encoding a putative ATP-dependent exporter of carboxylate anions, a zinc finger protein, and a potassium transporter, respectively. Of note, the GABA permease-encoding gene *UGA4* is, like its *A. nidulans gabA* orthologue, an acid-expressed gene (86). Also like *gabA, UGA4* is subject to complex regulation. For example, *UGA4* is repressed by the Dal80p/Uga43p GATA factor, whose transcription is also preferential under certain acidic conditions (86). In none of these cases has it been investigated whether their preferential acid expression is Rim101p dependent (i.e., involving Rim101p repression at alkaline pH), although in the case of *UGA4* a consensus PacC/Rim101p binding site is found 237 bp upstream of its coding region (86). *UGA4* is probably a good example of a case where profiling studies have failed to detect pH regulation because the inducer (GABA) was absent from the growth media (although some inducer is possibly available intracellularly from intermediary metabolism).

Molecular Characterization of Components of the *RIM (pal)* Signaling Pathway

In addition to *RIM101*, the *S. cerevisiae* genome contains homologues for every *pal* gene except *palC* (Table 2) (48, 56, 68, 90, 91, 124, 135).

Two potential PalA homologues, Bro1p and Rim20p, are found in the yeast genome (90), but only the latter belongs to the *RIM* pathway (135). Two-hybrid and coimmunoprecipitation analyses revealed that Rim20p is able to bind Rim101p, whose residues 547 to 678 suffice for reasonably strong interaction with Rim20p, although a second, nonoverlapping region (residues 297 to 546) mediates a weak interaction (135). PalA also interacts with PacC (Vincent and Peñalva, unpublished). Genome-wide two-hybrid analysis (60) revealed that Rim20p and Rim13p interact with Snf7p/Vps32p, the product of a class E gene involved in vacuolar biogenesis. While the possible

relationship of endocytosis with pH signaling is described in the *Yarrowia* section (see below), this ménage à trois prompted Xu and Mitchell (135) to hypothesize, in view of the ability of Rim20p to bind a C-terminal region of Rim101p, that Snf7p might act as an adapter through which Rim20p/PalA would recruit Rim13p/PalB to its putative target in the C-terminal region of Rim101p/PacC (135). While this model agrees with available evidence, it has not yet been established whether the Rim20p interaction with Rim101p requires another yeast protein.

Another important feature of the *RIM* pathway genes concerns *RIM9*, the *palI* homologue. As described above, *palI*Δ mutants are exceptional in that they grow to some extent on alkaline pH plates. Both Rim9p (71) and PalI (31) contain four predicted transmembrane domains within a region of high amino acid sequence conservation, but they are markedly different in size (Rim9p, 239 residues; PalI, 549 residues; the revised sequence is given in the addendum in reference 31), which is accounted for by the presence in PalI of a ~370-residue hydrophilic, putatively cytosolic tail which is absent from Rim9p (31). PalI and Rim9p have been suggested to be membrane sensors (31). It will be interesting to see if the differences in the C termini of the two proteins affect their efficiencies or mode of action.

RIM13 (117), the *palB* homologue (Table 2), was also identified independently and denoted *CPL1* because it encodes a calpain-like protein containing the diagnostic Pal homology domain (114) (see above) and the essential (as determined by site-directed mutagenesis) Cys128 residue, which almost certainly provides the catalytic thiol (48). PalH homologue Rim21p was identified by Tréton et al. (124), who also corrected a sequencing error in the yeast genome sequence, leading to the identification of *RIM8/palF*, which had been previously identified genetically (117).

pH REGULATION IN *CANDIDA ALBICANS*

Dimorphism and Pathogenesis

Candida spp. are the fourth-leading causes of nosocomial infection worldwide (18). *C. albicans*, the most prevalent fungal pathogen of humans, is a dimorphic fungus which undergoes reversible morphological transitions between unicellular yeast-like and hyphal and pseudohyphal growth forms in response to environmental signals, of which those involving ambient pH and temperature changes have been best characterized. Under optimal (37°C) temperature conditions, filamentation is favored by ambient pHs close to neutrality and is considerably reduced at pHs lower than 6. In contrast, the yeast form predominates almost exclusively at pH 4 (13). The PacC (*CaRIM101/PPR2* in this organism) pH regulatory system is conserved in *Candida* spp. and plays a major role in controlling the pH-dependent dimorphic transition. The ability of *C. albicans* to undergo the dimorphic transition and to respond to diverse environments is thought to be essential for pathogenesis, and, at least in mouse models of systemic infection, mutants impaired in filamentation are largely avirulent (73). Therefore, the mechanisms by which pH regulation controls *Candida* dimorphism and adaptation to different ambient pH niches within the infected host have received considerable attention. Readers interested in *Candida* virulence factors and

dimorphism can consult recent excellent reviews (8, 8a, 38, 72a, 85). A recently published thorough review of fungal development and virulence (70) is strongly recommended to complement this account of pH regulation.

Characterization of the pH-Dependent Morphological Transition

The molecular characterization of the pH-regulated transition in *Candida* was pioneered by Fonzi's group, who used differential screening techniques to isolate *PHR1*, a prototypical alkali-expressed gene encoding a 548-residue membrane-anchored glycosylphosphatidylinositol cell surface protein whose expression is prevented by acidic pH. Phr1p and its Phr2p homologue (see below) are 1,3-β-glucanosyltransferases involved in fungal cell wall synthesis (44, 87). At alkaline pH (where *PHR1* is expressed and the filamentous mode of growth predominates), a homozygous *phr1*Δ mutant showed morphological abnormalities resulting from its inability to grow apically (108). Since this mutant grew normally under acidic pH conditions (where the yeast mode of growth predominates), the existence of a *PHR1* isofunctional homologue active at acidic pH was predicted and eventually confirmed (88). This gene was designated *PHR2* (88). *PHR2* is a prototypical acid-expressed gene showing a pattern of expression inverse to that of *PHR1*. A *phr2*Δ mutant showed morphological abnormalities and was unable to grow at acidic pH but grew normally under filamentous growth-promoting alkaline pH conditions (88). Therefore, *PHR1* and *PHR2* encode isofunctional, mutually interchangeable proteins having a key role in the adaptation of *Candida* to alkaline and acidic growth conditions, respectively.

C. albicans is able to cause infections in a broad range of host niches which show significant differences in ambient pH. For example, the mouse systemic pH is ~7.3 whereas the pH in the rat vagina is ~4.5. This suggested that the ability of *Candida* to react appropriately to (among other environmental variables) rather different pH environments is crucial for its pathogenicity. Null *phr1*Δ and *phr2*Δ strains provided the first evidence for this proposal. A *phr1*Δ mutant is avirulent in a systemic mouse model of candidiasis (29, 53), whereas a *phr2*Δ mutant is virulent. In contrast, the *phr2*Δ mutant is strongly attenuated in virulence in a rat vaginitis model whereas the alkali-expressed *PHR1* gene is dispensable for infection of the vaginal niche (29). Therefore, the involvement of pH regulation in infection shown by this work is buttressed by the fact that the virulent phenotype parallels the in vitro pH dependence of the mutant strains (29).

Characterization of the Genes Involved in pH Regulation

The analysis of pH regulation in *A. nidulans* and its conservation in *S. cerevisiae* strongly suggested that the reverse modes of expression of *PHR1* and *PHR2* reflect their regulation by a *C. albicans* pH regulatory system involving homologues of *pacC* and the *pal* genes. The Fonzi (104) and Mitchell (28) groups identified the *C. albicans pacC* homologue, designated *CaRIM101/PRR2*. We will use here the *RIM101* designation (preceded by *Ca*, when needed, to indicate *C. albicans*), in agreement with the use of *S. cerevisiae* gene nomenclature to

designate *C. albicans* homologues, rather than the *PRR2* (for "pH Response Regulator") designation (104). *CaRIM101* encodes a 661-residue polypeptide (note that the ORF reported in reference 28 is missing the N-terminal 58 codons of the complete ORF [104]). PacC, CaRim101p, and ScRim101p have extensive amino acid sequence identity in their tridactyl zinc finger regions but otherwise diverge substantially.

Northern analysis of *PHR1* and *PRA1* (another alkali-expressed gene) (111) in a *Carim101Δ* strain confirmed that under alkaline growth conditions, *PHR1* and *PRA1* are subject to the positive action of CaRim101p whereas *PHR2* is repressed by this PacC homologue (28, 104). Neither *PHR2* repression nor *PHR1* (and *PRA1*) activation takes place under acidic pH conditions. Therefore, CaRim101p, like PacC (122), is required for repression of acid-expressed genes and for activation of alkali-expressed genes. Additionally, like *pacC*, *CaRIM101* behaves as an alkali-expressed gene subject to its own positive control (28, 104).

The characterization of two *C. albicans pal* genes, *CaRIM8/PPR1* (the *palF* homologue) and *CaRIM20* (the *palA* homologue), has been reported (28, 99). Like *Carim101Δ* mutations, homozygous *Carim8Δ* (28, 99) and *Carim20Δ* mutations (28) lead to acidity mimicry, as shown by elevated *PHR2* expression and absence of *PHR1* expression at both pH 4.0 and pH 7.5, i.e., in a pH-independent manner. This is in complete agreement with the *A. nidulans* model in Fig. 1 and demonstrates that a pH signaling pathway isofunctional to the *pal* pathway operates in *C. albicans*. Indeed, putative *palB, palH,* and *palI* (but not *palC*) homologues are present in the *C. albicans* genome (http://genolist.pasteur.fr/CandidaDB/). *CaRIM8* appears to be an acid-expressed gene (99), although it remains to be determined whether it is under the negative action of *CaRIM101*.

Involvement of pH Regulatory Genes in Filamentation and Virulence

Since the absence of *CaRIM101* function leads to acidity mimicry, it would be expected that filamentous growth, which is favored by alkaline growth conditions, should be impaired in a *Carim101Δ* strain. In agreement, a *Carim101* allele compromised the ability of cells to filament on spider medium (133). The *Carim101Δ* allele constructed by Ramón et al. (104) truncates Rim101p upstream of the DNA binding domain and almost certainly represents a null allele. A homozygous *Carim101Δ* strain was completely deficient in filamentation on both agar-solidified pH 7.5 199 medium and 10% serum medium and was largely defective on spider medium (104). In liquid 199 medium adjusted to pH 7.5, homozygous *Carim101* and *Carim101Δ* strains were unable to filament. However, this morphogenetic defect was not observed on incubation in serum, which promotes filamentation (133). All the above data show that although *CaRIM101* plays a major role in filamentation, it is not essential to filamentation under all growth conditions.

In agreement with the model in Fig. 1, the acidity-mimicking *Carim20Δ* and *Carim8Δ* mutations in homozygosis also prevent filamentation on several (but not on all) growth media (28, 99, 133). Forced expression of *PHR1* did not restore the filamentation defect of a *Carim8Δ* mutant, indicating that other

CaRIM101-dependent, alkali-expressed genes are also required for hyphal development (and/or that expression of a *CaRIM101*-dependent acid-expressed gene[s] is preventing filamentation) (99).

Since acidity-mimicking *Carim101, Carim8* or *Carim20* homozygotes are impaired in filamentation and deficient in the pH response, it was predicted that these strains should also be largely avirulent. That the *CaRIM101* pathway is required for pathogenesis in vivo was confirmed in a mouse model of systemic candidiasis, where these mutants showed significant reductions in virulence and development of kidney pathology compared to the wild type and were less damaging to endothelial cells, a process thought to precede the invasion of underlying tissues (27). A dominant gain-of-function truncating mutation in *CaRIM101* resembling *pacC^c* alkalinity-mimicking mutations (28) rescued all virulence defects resulting from the *Carim8* homozygous mutation and was thus shown to be epistatic in the mouse model to a mutation inactivating the *pal* pathway (27). This establishes that the only function of CaRim8p in infection takes place through the *CaRIM101*-dependent regulation of genes involved in pathogenesis.

Alkalinity-Mimicking Mutations Truncating CaRim101p

CaRIM101-405 is a constructed alkalinity-mimicking allele (28) truncating the protein (and the mRNA) after Asn462. This allele is epistatic to *Carim8* and *Carim20* mutations with respect to filamentation defects, but the complete epistasis seen under alkaline conditions is only partial under acidic conditions, which indicates that double-mutant strains are still to some extent pH dependent for filamentation. It is difficult to judge the significance of this finding, since it has not yet been established whether CaRim101p, like its *A. nidulans* and *S. cerevisiae* homologues, is activated by proteolytic processing and, if so, what is the approximate C-terminal residue of the putative processed form. Genetic evidence (outlined below), however, strongly suggests that CaRim101p is activated by a processing reaction removing a negative-acting C-terminal domain. Moreover, as seen with certain *YlRIM101* alleles (see below), mutational truncation of the mRNA might lead to mRNA instability, possibly obscuring the mutant protein phenotype.

A genetic approach to isolating dominant, alkalinity-mimicking mutations in *CaRIM101* has also been used (37). Homozygous *phr2Δ* strains do not grow at acidic pH. Suppressors of this conditional growth defect result from dominant *CaRIM101* mutations promoting pH-independent expression of *PHR1*, which compensates for the loss of *PHR2* (37). In heterozygosis, these mutations also confer the ability to filament at acidic pH (37). *CaRIM101-1426* (Gln476stop) removes 186 residues from the C terminus of CaRim101p, whereas *CaRIM101-1751* (Ser584stop) removes 78 residues (37). These dominant alleles result in the ability to filament at acidic pH, pH-independent expression of *PHR1*, and no expression of *PHR2* at any ambient pH.

The dominant *CaRIM101-1426* allele was unable to bypass the filamentation defect of an *efg1Δ* mutant strain, either alone or in combination with a *cph1Δ* mutation, but was able to suppress the weaker filamentation deficiency phenotype of a

single *cph1Δ* mutation (37). These data show that although CaRim101p plays a prominent role in controlling hyphal formation, it does not do so independently of other regulatory proteins. Efg1p (116) is homologous to the *S. cerevisiae* transcription factor Pdh1p, whose overexpression promotes pseudohyphal growth (55), and to *A. nidulans* StuA, which is involved in pseudohypha-like growth during conidiophore development (36). Efg1p inactivation largely restricts filamentation (73, 116). Efg1p cooperates with *C. albicans* Cph1p (the *S. cerevisiae* Ste12p homologue) to promote filamentous growth, and a *C. albicans cph1/cph1 efg1/efg1* double mutant is avirulent and unable to form hyphae or pseudohyphae in response to many stimuli (73).

Porta et al. (100) isolated extragenic dominant suppressors of a *Carim8Δ* (*prr1Δ*) homozygous mutation leading to a filamentation defect (see above). These were identified as sectors of filamentous growth projecting from the otherwise smooth-bordered nonfilamentous mutant colonies. All eight suppressors thus isolated were found to be *CaRIM101* nonsense mutations truncating CaRim101p after residues 557 through 586, thereby removing between 75 and 104 residues from the C terminus of the protein (100). In their alkalinity mimicry, these suppressors resemble *pacC^c*-truncating mutations. Taken together with the above data on truncating mutants (37), these data show that CaRim101p contains a pH-dependent, C-terminal negative-acting region and are consistent with C-terminal proteolytic processing activation of CaRim101p, underscoring the strong parallels between PacC and CaRim101p regulation (100).

Mühlschlegel and colleagues (F. Mühlschlegel, personal communication) constructed a series of CaRim101p truncating mutations by replacing codons 281, 305, 333, 385, 411, and 463 with nonsense codons. Mutants truncated after residues 280, 304, and 332, located within or immediately C-terminal to the zinc finger region, are unable to suppress the *phr2Δ* mutation for growth at pH 4, which strongly suggests that these three mutations represent the loss-of-function class. It would be interesting to know whether all or some of these severely truncated proteins are also unable to repress *PHR2*, as is the case for a complete CaRim101p deficiency. Truncation after residues 384, 410, and 462 restores growth at pH 4 in a *phr2Δ* background, which indicates that these proteins are competent to activate *PHR1* in a pH-independent manner. However, while a CaRim101p truncated after Asn462 is fully competent to promote filamentation at acidic pH, a mutant truncated after residue 410 is not. This strongly suggests that sequences within residues 411 through 462 are essential to promote filamentation by the activated Rim101p form but not to activate *PHR1* expression (Mühlschlegel, personal communication).

pH Regulation in *Candida dubliniensis*

C. dubliniensis is a dimorphic yeast which is phylogenetically related to *C. albicans* but which appears to be a less aggressive pathogen. *C. dubliniensis* causes disease mostly in human immunodeficiency virus-infected immunocompromised individuals, in which it leads to oropharyngeal candidiasis (58). This yeast has *PHR1* and *PHR2* homologues which are expressed in a pH-dependent manner (58) and contains a *CaRIM101*-de-

pendent pH regulatory circuit (Mühlschlegel, personal communication).

pH REGULATION IN *YARROWIA LIPOLYTICA*

Regulation of Protease Gene Expression by Ambient pH

In *Y. lipolytica*, transcription of the *XPR2* and *AXP1* genes, encoding alkaline (Aep) and acidic (Axp) extracellular proteases, respectively, is regulated by ambient pH. This has been exploited by Gaillardin and colleagues to characterize the pH-regulatory system of this dimorphic yeast (26, 69, 124).

With appropriate carbon and nitrogen nutrition, *XPR2* is expressed at pH 6.8 or above and its transcription is completely prevented under acidic pH conditions. Therefore, *XPR2* can be viewed as a prototypical "alkaline" gene, as defined in the *A. nidulans* system. Recessive mutations in four different loci designated *PAL1, PAL2, PAL3,* and *PAL4* prevent expression of the alkaline protease at pH 6.8 and therefore result, at least partially, in acidity mimicry. As discussed below, *PAL2* and *PAL3* turned out to be the *Yarrowia* homologues of *palH* and *palF*, respectively (124). A recent insertional mutagenesis study (56) identified homologues for all other *pal* genes, with the sole exception of *palC*, which appears to be absent from *Yarrowia*, in agreement with its apparent absence from the *Candida* and *Saccharomyces* genomes. Finally, a dominant, gain-of-function *RPH2* mutation in a sixth pH regulatory locus (69) turned out to be a mutation in *YlRIM101*, which encodes a 585-residue protein with 35% identity to PacC and a somewhat lower identity (28%) to *S. cerevisiae* Rim101p. Like its homologues, YlRim101p contains three canonical Cys$_2$His$_2$ zinc fingers with very high amino acid sequence identity to other members of the PacC/Rim101p family. As occurs with other members of the family, the degree of sequence identity is substantially lower outside the nearly N-terminally located DNA binding domain.

Mutations in *pal* Genes

The phenotype of the classical *pal2* (*YlRIM21*) and *pal3* (*YlRIM8*) alleles was indistinguishable from that of the constructed null alleles (69, 124), confirming that, as in *A. nidulans*, these acidity-mimicking mutations in the *pal* signaling pathway genes represent the loss-of-function class. In contrast to *A. nidulans*, however, these mutations as well as those resulting from insertional inactivation of *YlRIM9/palI, YlRIM20/palA,* and *YlRIM13/palB*, which prevent expression of the alkaline genes at alkaline pH, did not derepress the acidic *AXP1* gene (69). In common with a *Ylrim101Δ* allele, *pal* mutations affected mating efficiency and sporulation, although the YlRim101p requirement appeared to be more stringent (69) (see below). In contrast to *Saccharomyces, pal* mutations do not prevent invasiveness (124); in contrast to *Candida*, they do not impair hyphal formation (although hyphal branching frequency is increased) (56, 120, 124). Recent data (120) strongly suggest that the known pH dependency of the transition from the yeast to the hyphal mode of growth in *Yarrowia* reflects the indirect control by ambient pH of the availability of organic nitrogen sources in complex media, which would explain the absence of morphogenetic defects resulting from pH-regula-

tory mutations. Mutations in *YlRIM13, YlRIM20*, and *YlRIM9* impaired growth at alkaline pH, but *palI* mutations, like *palI* mutations, showed a much less pronounced effect (56).

Mutations in *YlRIM101*

The fact that a dominant gain-of-function mutation suppressed the acidity mimicry of *pal* mutations with respect to *XPR2* expression was the basis for the identification of *YlRIM101* (69). The molecular characterization of a *YlRIM101*-containing plasmid revealed that the construction resulted in premature *YlRIM101* truncation after codon 473. Therefore, as in PacC, a C-terminal region negatively modulates YlRim101p in a *pal* pathway-dependent manner, as shown by epistasis of the above truncating mutation to *pal⁻* alleles. As predicted by the PacC model, a null *Ylrim101Δ* mutation leads to acidity mimicry. Like *pal⁻* mutations, *Ylrim101Δ* prevents the expression of both the acidic and the alkaline protease genes, impairs growth at alkaline pH, and has no effect on invasiveness and only a minor effect on hyphal formation (56, 69, 120).

The finding that mutations inactivating either *YlRIM101* or the *pal* pathway, all of which classify as Aep⁻ Axp⁻ in reporter tests, did not result in derepression of an acid-expressed gene such as *AXP1* was unexpected in view of the *Aspergillus* results. This paradoxical result probably reflects the complex mode of regulation of the *AXP1* acidic protease gene, which is regulated by carbon and nitrogen sources as well as by specific induction by peptides. This complex regulation was evidenced in an insertional mutagenesis scan for genes affecting the expression of these proteases (56), which identified, in addition to pH-regulatory genes, other genes encoding, for example, the Opt1p oligopeptide transporter and Ssy5p, a component of a sensing complex which in budding yeast signals the presence of extracellular amino acids (56). *opt1* or *ssy5* mutations do not impair pH-regulatory phenotypes other than expression of the protease genes and therefore most probably affect a pathway required for protease induction. Thus, the reason why YlRim101p inactivation does not lead to *AXP1* expression under alkaline conditions might be that protease-specific inducing conditions may not be achievable at alkaline pH (if, for example, the "acidic" Axp enzyme were involved in producing oligopeptides acting as specific inducers, as suggested by González-López et al. [56]). In agreement with the above interpretation, it was shown that the *Yarrowia* homologue of *S. cerevisiae YMR110c*, a gene which is preferentially expressed at acidic pH, is unaffected by *Ylrim* mutations at acidic pH but clearly derepressed at alkaline pH, especially in a *Ylrim101Δ* background (56). Therefore, in contrast to the *AXP1* gene, *YMR110c* is a prototypical acid-expressed gene, in agreement with the model in Fig. 1.

The phenotype of extant *YlRIM101* gain-of-function mutations bypassing the *pal* pathway is heterogeneous, as expected from the direct involvement of its product in pH regulation (see below). This also reflects the major effects of truncating the *YlRIM101* mRNA on transcript stability. A reduction in YlRim101p levels (e.g., through reduced transcript stability) will have an opposing effect (i.e. partial loss of function) to the gain of function resulting from C-terminal truncation of YlRim101p (69). *YlRIM101-1116* encodes a protein (and a transcript) truncated after residue 473, leading to very low

mRNA levels (69). mRNA instability possibly explains why this allele leads to only partial elevation of *XPR2* transcript levels at acidic pH (i.e., *XPR2* transcription is still partially pH dependent). A second constructed allele, *YlRIM101-1119*, encodes a YlRim101p truncated after residue 330 (69), a position which would be the approximate YlRim101p equivalent to the processing site in PacC. The resulting transcript is also unstable, but the activating functions of this mutant protein would appear to be less highly preserved (as determined by the only partially pH-independent *XPR2* expression) than the repressing functions (as determined by the complete absence of *AXP1* expression under acidic conditions) (69). In contrast to the above mutations leading to mRNA instability, *YlRIM101-5* (encoding a YlRim101p truncated after residue 419 by introducing a stop codon into the otherwise wild-type mRNA) does not affect transcript stability and leads to high, pH-independent *XPR2* transcript levels and full pH-independent repression of *AXP1* (69). Therefore, the behavior of dominant *YlRIM101* mutations approximates that of *pacC^c* mutations and is consistent with repression of acidic genes and activation of alkaline genes by the transcription factor.

YlRim101p Binding Site

The in vivo role of the YlRim101p binding site(s) in the *XPR2* promoter has been confirmed by dimethyl sulfate footprinting analysis (7). The *XPR2^p* UAS2 contains within a direct decameric repeat located at positions −146 through −127 a double direct repeat of a consensus PacC binding site, as defined by Espeso et al. (43), which enhances transcriptional activity when transplanted to a UAS2-deficient promoter (7, 78). The positive-acting role of this repeat on *XPR2* transcription is abolished in a null *YlRIM101* background but is active in a pH-independent manner in a *YlRIM101-1119* strain, demonstrating a direct activating role of the transcription factor on this alkaline gene (78). However, this pH-independent positive action is abolished in the presence of preferred carbon and nitrogen sources, which strongly suggests that other proteins may compete with Rim101p binding under such conditions to shut off *XPR2* transcription, whatever the ambient pH (78). This is reminiscent of the situation in the *A. nidulans* GABA permease gene promoter, where PacC, acting as a repressor, competes for binding with the IntA/AmdR pathway-specific activator at overlapping sites (39).

Possible Role of Plasma Membrane-to-Vacuole Endocytosis

The random insertional mutagenesis analysis of *AXP1* and *XPR2* expression (56) revealed that the effects of inactivating mutations in the *Y. lipolytica VPS28* gene on protease expression are very similar to those of *pal⁻* or *Ylrim101⁻* mutations. *S. cerevisiae VPS28* is a class E VPS (for "Vacuolar Protein Sorting") gene. Class E VPS genes mediate trafficking out of the prevacuolar compartment, where the vacuolar biogenesis and the endocytotic pathways converge, into the vacuole. Mutants with mutations in any of the class E genes accumulate an enlarged version of the prevacuolar compartment (12). Vps28p is, with class E products Vps23p and Vps37p, a member of the endosomal sorting complex ESCRT-1, whose function is required for sorting of endocytosed cargo into the multivesicular

body vesicles forming in the late endosome (63). The possible role of Vps28p in pH regulation merits consideration, since *Ylvps28* mutants share phenotypic characteristics of *Ylopt1* and *Ylssy5* mutants (56, 120) and thus Vps28p might be involved in the specific induction of the protease genes rather than in pH regulation. However, at least two observations had previously pointed toward the possible involvement of membrane-to-vacuole endocytotic trafficking in the pH-regulatory circuitry. First, two-hybrid screens involving the complete yeast proteome (60, 125) revealed that both Rim13p/PalB and Rim20p/PalA interact with Snf7p/Vps32p, another class E Vps protein. Moreover, the Vps4p AAA ATPase, another class E product which interacts with and regulates the membrane association of Snf7p (5), also interacts with Rim20p. Secondly, Bro1p (92) is another *S. cerevisiae* Rim20p/PalA-like homologue (90) which is not involved in pH regulation (135). Bro1p has been isolated from cell extracts in a complex with Snf7p/Vps32p and TAP-tagged Vps4p (52), possibly validating ex vivo data for Rim20p interactions. Therefore, both Rim20p/PalA and its Bro1p possible homologue have been shown to form complexes with Vps32p and Vps4p, suggesting that these putative homologues share the ability to interact with these "endocytotic" proteins. In agreement with a role for Bro1p in endocytosis, *bro1* mutations were among recessive mutations affecting several genes controlling different aspects of post-Golgi endosomal-vacuolar protein sorting, selected as suppressing mutations inactivating the yeast Ssy1p-Ptr3p-Ssy5p (STS) plasma membrane amino acid sensor complex (45). (It should be recalled that Ssy5p plays a critical role in regulating *Y. lipolytica AXP1* and *XPR2* expression [see above].) The possible role of endocytosis in pH regulation is an open question, and, as speculated by Xu and Mitchell (135), it is possible that either the ambient pH membrane receptor is active only after its endocytosis or the acidification of endocytosed fluid is the actual signal by which cells measure ambient pH.

pH REGULATION: SOME PRACTICAL APPLICATIONS

Antibiotic Production

Alkaline growth conditions and alkalinity-mimicking *pacC^c* mutations substantially increase penicillin production in the model organism *A. nidulans* (42, 112), where *acvA, ipnA*, and *acyA*, the three structural genes required for penicillin biosynthesis from precursor amino acids, are under the positive action of PacC (42, 97, 121; E. A. Espeso and M. A. Peñalva., unpublished data). (The direct control of the *ipnA* promoter by PacC has been discussed above.) The positive action of PacC-mediated pH regulation overrides the negative effects of a repressing carbon source, although pH and carbon regulation normally act in concert, since poor derepressing carbon sources lead to external alkalinization, possibly because they favor the utilization of the carbon skeleton of amino acids with subsequent release of ammonia (42). These findings stimulated the characterization of *pacC* in the industrially important producer *P. chrysogenum* (119), whose product also recognizes in vitro the GCCARG PacC consensus (43, 122) in the bidirectional promoter of the *acvA-ipnA P. chrysogenum* homologues *pcbAB-pcbC* (119). It has been demonstrated that *P. chrysoge-*

num pacC mutations equivalent to alkalinity-mimicking *pacC^c* mutations indeed result in penicillin overproduction (T. Suárez, H. N. Arst, Jr., G. Turner, and M. A. Peñalva, international patent application PCT/ES00/00464). The details of regulation, however, differ in the two organisms because alkaline pH does not override the negative effects of a repressing carbon source in *P. chrysogenum* (119). The finding that tailored *pacC* mutations can improve β-lactam antibiotic yields in an industrially useful antibiotic producer might be very important commercially because transcript level determinations and gel retardation experiments indicate that pH regulation is also likely to play an important role in cephalosporin (another β-lactam antibiotic) production by the industrially important *Acremonium chrysogenum* (110).

The antifungal protein AFP secreted by *Aspergillus giganteus* (67) is a 51-residue polypeptide antibiotic which inhibits the growth of a number of filamentous fungi. This remarkably stable protein (67), folded in a five-stranded compact β-barrel stabilized by four internal disulfide bridges (19), promotes the aggregation of acidic phospholipid vesicles (67). The *afp* gene has been recently shown to be transcriptionally regulated by ambient pH, showing a prototypical alkaline gene expression pattern, which suggests that it is under PacC control, in agreement with the presence of two putative PacC binding sites within its 5′ upstream region (80).

Prevention of Toxin Production

Production of aflatoxin by *Aspergillus parasiticus* is highest under acidic growth conditions, as is production of sterigmatocystin, an aflatoxin precursor and a toxin in its own right, by *A. nidulans* (64). An alkalinity-mimicking *pacC^c* mutation drastically reduces sterigmatocystin production (64).

Enzyme Production

Commercially important secreted enzymes subject to pH regulation offer obvious potential for exploiting the response to ambient pH. *A. niger* is an important producer of extracellular enzymes, many of which are subject to *pacC*-mediated pH regulation (61, 76, 126, 127). In another example, ambient pH influences both the production and glycosylation of *A. niger* glucoamylase (131). The promoter of the *Aspergillus tubingensis* xylanase-encoding gene *xlnA* (30) merits mention. This promoter is strictly dependent on xylan induction mediated through three target heptanucleotides for the pathway-specific zinc binuclear cluster transcription factor XlnR (129). Each of these sites is located within a 14-bp repeat and is overlapped by consensus (repeats 2 and 3) or near-consensus (repeat 1) PacC binding sites (30). This situation is remarkably similar to that found in the acid-expressed *gabA* promoter of *A. nidulans* (39), which would suggest that PacC here also prevents the expression of a structural gene by competing with the pathway-specific activator for target site binding. However, although the regular arrangement and overlapping of XlnR and PacC binding sites in this promoter almost certainly indicate control by PacC, it remains to be established whether *A. tubingensis xlnA*, like *A. nidulans xlnA* and *xlnB*, is pH regulated.

The use of pH-regulated promoters for protein production by fungi is an equally obvious target for exploitation. For ex-

ample, loss-of-function, acidity-mimicking *palB* mutations enhance the production of a *Thermomyces lamuginosus* lipase expressed in *Aspergillus oryzae* (137) and an alkaline protease promoter has been shown to be useful to drive the synthesis of an extracellular enzyme under alkaline conditions (132).

Targets for Preventing Animal Pathogenicity

The involvement of the pH-regulatory system in fungal pathogenicity of animals was unknowingly demonstrated by using *A. nidulans* three decades ago (103). Compared to the wild type, strains carrying a *palA, palB*, or *palF* mutation showed reduced virulence after inoculation into mice. A *pacC^c* mutation had no effect on virulence, and the reduced virulence associated with a *palB* mutation was hypostatic to the virulence of a *pacC^c* mutation in a double mutant, consistent with the epistasis of *pacC^c* mutations to pH signal transduction *pal* mutations seen in biochemical and physiological tests. The thoroughly studied involvement of the pH-regulatory system in virulence of *C. albicans* is described above.

Ambient pH also appears to be an important signal controlling the levels of expression of a number of virulence determinants in the insect pathogen *Metarhizium anisopliae*, where expression levels change in concert with rising pH during the infection process (115). This example also suggests that the release of ammonia by fungi under certain limiting carbon and nitrogen source conditions may serve to tailor the pH of the microbial microenvironment to values at which extracellular activities are most efficient or become most useful, as suggested for penicillin production, where antibiotic excretion might help the fungal colony to compete for nutrients under conditions of carbon limitation leading to ambient pH alkalinization (42).

Targets for Preventing Plant Pathogenicity

pH regulation is likely to be an important determinant in fungal phytopathogenicity. Acidic ambient pH is important for the establishment of plant infections by *Sclerotinia sclerotiorum*, and the expression of *acp1*, encoding a non-aspartyl acid protease, and *pg1*, encoding an endopolygalacturonase, both likely to play important roles in infection, is elevated under acidic conditions (101, 106). Sclerotial development, necessary for long-term survival, also requires acidic conditions (106). A *pacC* homologue is, like *A. nidulans pacC*, alkali expressed, and the ambient pH regulatory system has been proposed to play an important role in pathogenicity (106). In contrast, pathogenicity by *Colletotrichum gloeosporioides* and expression of a pectate lyase gene important for virulence are dependent on the rise in pH occurring during fruit ripening, which the fungus accelerates by secreting ammonia (102, 136).

The *pacC* gene of *Fusarium oxysporum*, a pathogen which causes vascular wilt disease in a wide variety of economically important crops, has been characterized and shown to be an alkali-expressed gene (A. Di Pietro, personal communication). As in *A. nidulans, F. oxysporum* strains carrying a loss-of-function mutation or a dominant, truncated gain-of-function *pacC^c* allele showed acidity-mimicking and alkalinity-mimicking phenotypes, respectively. The loss-of-function mutant grew poorly at alkaline pH, showed overexpression of acid-expressed polygalacturonase genes at acidic pH, and secreted increased levels of acid protease. In contrast, a mutant carrying the *pacC^c* allele had reduced acidic protease and increased alkaline protease activities. In infection assays with tomato plants, the loss-of-function mutant was as virulent as the wild-type strain whereas transformants carrying a *pacC^c* allele had significantly reduced virulence, suggesting that reduced virulence of the *pacC^c* mutant might result from inappropriately reduced expression of acidic genes important for infection (or, less likely, from elevated expression of alkaline genes) (Di Pietro, personal communication).

PERSPECTIVES

Those who pay the bill for the research described above (i.e., the public) will wish to know whether inhibitors of the pH regulatory system can be obtained and, if so, whether they will prevent fungal pathogenesis of animals (particularly humans) and/or plants. They might also wish to know whether such inhibitors will curtail contamination of foods by fungal toxins. The answers to these questions are likely to be forthcoming, and any affirmative responses are likely to be greatly beneficial.

Those who do the research and those who read about it in the scientific literature will wish, in the era of genomics and proteomics, to know the full extent of the range of activities subject to pH regulation. They might also wish to know the relationship between pH regulation and pH homeostasis, how the transcription factor-encoding genes *pacC/RIM101* are themselves regulated, and the identity of the processing protease. They will surely want to know precisely how ambient pH is sensed and how the signal is transmitted to PacC/Rim101p, a biologically novel process which we are only beginning to glimpse. Finally, they are likely to be fascinated by the presence of pH-regulatory system component homologues beyond the fungal kingdom. Is pH regulation the ancestral function, and what are the current functions of the homologues in other kingdoms?

REFERENCES

1. **Arst, H. N., Jr.** 1967. Studies on metabolic control and uptake in *Aspergillus*. Ph.D. thesis. University of Cambridge, Cambridge, United Kingdom.
2. **Arst, H. N., Jr., C. R. Bailey, and H. A. Penfold.** 1980. A possible role for acid phosphatase in γ-amino-n-butyrate uptake in *Aspergillus nidulans*. Arch. Microbiol. **125:**153–158.
3. **Arst, H. N., Jr., E. Bignell, and J. Tilburn.** 1994. Two new genes involved in signalling ambient pH in *Aspergillus nidulans*. Mol. Gen. Genet. **245:**787–790.
4. **Arst, H. N., Jr., and D. J. Cove.** 1970. Molybdate metabolism in *Aspergillus nidulans*. II. Mutations affecting phosphatase activity or galactose utilization. Mol. Gen. Genet. **108:**146–153.
5. **Babst, M., B. Wendland, E. J. Estepa, and S. D. Emr.** 1998. The Vps4p AAA ATPase regulates membrane association of a Vps protein complex required for normal endosome function. EMBO J. **17:**2982–2993.
6. **Bailey, C. R., H. A. Penfold, and H. N. Arst, Jr.** 1979. *cis*-dominant regulatory mutations affecting the expression of GABA permease in *Aspergillus nidulans*. Mol. Gen. Genet. **169:**79–83.

7. **Blanchin-Roland, S., R. Cordero-Otero, and C. Gaillardin.** 1994. Two up-stream activation sequences control the expression of the *XPR2* gene in the yeast *Yarrowia lipolytica*. Mol. Cell. Biol. **14:**327–338.

8. **Brown, A. J. P., and N. A. R. Gow.** 1999. Regulatory networks controlling *Candida albicans* morphogenesis. Trends Microbiol. **7:**333–338.

8a.**Brown, A. J. P., and N. A. R. Gow.** 2001. Signal transduction and morphogenesis in *Candida albicans*, p. 55–71. *In* R. J. Howard and N. A. R. Gow (ed.), The mycota, vol. VIII. Biology of the fungal cell. Springer-Verlag KG, Berlin, Germany.

9. **Brown, D. W., J. H. Yu, H. S. Kelkar, M. Fernandes, T. C. Nesbitt, N. P. Kelkar, T. H. Adams, and T. J. Leonard.** 1996. Twenty-five coregulated transcripts define a sterigmatocystin gene cluster in *Aspergillus nidulans*. Proc. Natl. Acad. Sci. USA **93:**1418–1422.

10. **Brown, M. S., and J. L. Goldstein.** 1999. A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood. Proc. Natl. Acad. Sci. USA **96:**11041–11048.

11. **Brown, M. S., J. Ye, R. B. Rawson, and J. L. Goldstein.** 2001. Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans. Cell **100:**391–398.

12. **Bryant, N. J., and T. H. Stevens.** 1998. Vacuole biogenesis in *Saccharomyces cerevisiae*: protein transport pathways to the yeast vacuole. Microbiol. Mol. Biol. Rev. **62:**230–247.

13. **Buffo, J., M. A. Herman, and D. R. Soll.** 1984. A characterization of pH-regulated dimorphism in *Candida albicans*. Mycopathologia **85:**21–30.

14. **Caddick, M. X.** 1986. Phosphatases of *Aspergillus nidulans*. Ph.D. thesis. University of Newcastle upon Tyne, Newcastle upon Tyne, United Kingdom.

15. **Caddick, M. X., and H. N. Arst, Jr.** 1986. Structural genes for phosphatases in *Aspergillus nidulans*. Genet. Res. **47:**83–91.

16. **Caddick, M. X., A. G. Brownlee, and H. N. Arst, Jr.** 1986. Phosphatase regulation in *Aspergillus nidulans*: responses to nutritional starvation. Genet. Res. **47:**93–102.

17. **Caddick, M. X., A. G. Brownlee, and H. N. Arst, Jr.** 1986. Regulation of gene expression by pH of the growth medium in *Aspergillus nidulans*. Mol. Gen. Genet. **203:**346–353.

18. **Calderone, R. A., and W. A. Fonzi.** 2001. Virulence factors of *Candida albicans*. Trends Microbiol. **9:**327–335.

19. **Campos-Olivas, R., M. Bruix, J. Santoro, J. Lacadena, A. Martínez del Pozo, J. G. Gavilanes, and M. Rico.** 1995. NMR solution structure of the antifungal protein from *Aspergillus giganteus*: evidence for cysteine pairing isomerism. Biochemistry **34:**3009–3021.

20. **Cao, L., L. Zhang, P. Ruiz-Lozano, Q. Yang, K. R. Chien, R. M. Graham, and M. Zhou.** 1998. A novel putative protein-tyrosine phosphatase contains a BRO1-like domain and suppresses Ha-ras-mediated transformation. J. Biol. Chem. **273:**21077–21083.

21. **Carafoli, E., and M. Molinari.** 1998. Calpain: a protease in search of a function? Biochem. Biophys. Res. Commun. **247:**193–203.

22. **Causton, H. C., B. Ren, S. S. Koh, C. T. Harbison, E. Kanin, E. G. Jennings, T. I. Lee, H. L. True, E. S. Lander, and R. A. Young.** 2001. Remodeling of yeast genome expression in response to environmental changes. Mol. Biol. Cell **12:**323–337.

23. **Che, S., H. M. El-Hodiri, C. F. Wu, M. Nelman-Gonzalez, M. M. Well, L. D. Etkin, R. B. Clark, and J. Kuang.** 1999. Identification and cloning of Xp95, a putative signal transduction protein in *Xenopus* oocytes. J. Biol. Chem. **274:**5522–5531.

24. **Che, S., M. M. Weil, L. D. Etkin, H. F. Epstein, and J. Kuang.** 1997. Molecular cloning of a splice variant of *Caenorhabditis elegans* YNK1, a putative element in signal transduction. Biochim. Biophys. Acta **1354:**231–240.

25. **Cohen, B. L.** 1980. Transport and utilization of proteins by fungi, p. 411–430. *In* J. W. Payne (ed.), Microorganisms and nitrogen sources. John Wiley & Sons, Inc., New York, N.Y.

26. **Cordero-Otero, R., and C. Gaillardin.** 1996. Dominant mutations affecting expression of pH-regulated genes in *Yarrowia lipolytica*. Mol. Gen. Genet. **252:**311–319.

27. **Davis, D., J. E. J. Edwards, A. P. Mitchell, and A. S. Ibrahim.** 2000. *Candida albicans RIM101* pH response pathway is required for host-pathogen interactions. Infect. Immun. **68:**5953–5959.

28. **Davis, D., R. B. Wilson, and A. P. Mitchell.** 2000. RIM101-dependent and-independent pathways govern pH responses in *Candida albicans*. Mol. Cell. Biol. **20:**971–978.

29. **De Bernardis, F., F. A. Mühlschlegel, A. Cassone, and W. A. Fonzi.** 1998. The pH of the host niche controls gene expression in and virulence of *Candida albicans*. Infect. Immun. **66:**3317–3325.

30. **de Graaff, L. H., H. C. van den Broeck, A. J. J. van Ooijen, and J. Visser.** 1994. Regulation of the xylanase-encoding *xlnA* gene of *Aspergillus tubigensis*. Mol. Microbiol. **12:**479–490.

31. **Denison, S. H., S. Negrete-Urtasun, J. M. Mingot, J. Tilburn, W. A. Mayer, A. Goel, E. A. Espeso, M. A. Peñalva, and H. N. Arst, Jr.** 1998. Putative membrane components of signal transduction pathways for ambient pH regulation in *Aspergillus* and meiosis in *Saccharomyces* are homologous. Mol. Microbiol. **30:**259–264. (Addendum, **39:**211, 2001.)

32. **Denison, S. H., M. Orejas, and H. N. Arst, Jr.** 1995. Signaling of ambient pH in *Aspergillus* involves a cysteine protease. J. Biol. Chem. **270:**28519–28522.

33. **Díez, E., J. Álvaro, E. A. Espeso, L. Rainbow, T. Suárez, J. Tilburn, H. N. Arst, Jr., and M. A. Peñalva.** 2002. Activation of the *Aspergillus* PacC zinc-finger transcription factor requires two proteolytic steps. EMBO J. **21:**1350–1359.

34. **Dorn, G.** 1965. Genetic analysis of the phosphatases in *Aspergillus nidulans*. Genet. Res. **6:**13–26.

35. **Dorn, G.** 1965. Phosphatase mutants in *Aspergillus nidulans*. Science **150:**1183–1184.

36. **Dutton, J. R., S. Johns, and B. L. Miller.** 1997. StuAp is a sequence-specific transcription factor that regulates developmental complexity in *Aspergillus nidulans*. EMBO J. **16:**5710–5721.

37. **El Barkani, A., O. Kurzai, W. A. Fonzi, A. Ramón, A. Porta, M. Frosch, and F. A. Mühlschlegel.** 2000. Dominant active alleles of *RIM101 (PRR2)* bypass the pH restriction on filamentation of *Candida albicans*. Mol. Cell. Biol. **20:**4635–4647.

38. **Ernst, J. F.** 2000. Transcription factors in *Candida albicans*—environmental control of morphogenesis. Microbiology **146:**1763–1774.

39. **Espeso, E. A., and H. N. Arst, Jr.** 2000. On the mechanism by which alkaline pH prevents expression of an acid-expressed gene. Mol. Cell. Biol. **20:**3355–3363.

40. **Espeso, E. A., and M. A. Peñalva.** 1996. Three binding sites for the *Aspergillus nidulans* PacC zinc-finger transcription factor are necessary and sufficient for regulation by ambient pH of the isopenicillin N synthase gene promoter. J. Biol. Chem. **271:**28825–28830.

41. **Espeso, E. A., T. Roncal, E. Diez, L. Rainbow, E. Bignell, J. Álvaro, T. Suárez, S. H. Denison, J. Tilburn, H. N. Arst, Jr., and M. A. Peñalva.** 2000. On how a transcription factor can avoid its proteolytic activation in the absence of signal transduction. EMBO J. **19:**719–728.

42. **Espeso, E. A., J. Tilburn, H. N. Arst, Jr., and M. A. Peñalva.** 1993. pH regulation is a major determinant in expression of a fungal penicillin bio-synthetic gene. EMBO J. **12:**3947–3956.

43. **Espeso, E. A., J. Tilburn, L. Sánchez-Pulido, C. V. Brown, A. Valencia, H. N. Arst, Jr., and M. A. Peñalva.** 1997. Specific DNA recognition by the *Aspergillus nidulans* three zinc finger transcription factor PacC. J. Mol. Biol. **274:**466–480.

44. **Fonzi, W. A.** 1999. *PHR1* and *PHR2* of *Candida albicans* encode putative glycosidases required for proper cross-linking of beta-1,3- and beta-1,6-glucans. J. Bacteriol. **181:**7070–7079.

45. **Forsberg, H., M. Hammar, C. Andreasson, A. Moliner, and P. O. Ljungdahl.** 2001. Suppressors of *ssy1* and *ptr3* null mutations define novel amino acid sensor-independent genes in *Saccharomyces cerevisiae*. Genetics **158:**973–988.

46. **Foster, J. W.** 1999. When protons attack: microbial strategies of acid adaptation. Curr. Opin. Microbiol. **2:**170–174.

46a.**Foster, J. W.** 2000. Microbial responses to acid stress, p. 99–115. *In* G. Storz and R. Hengge-Aronis (ed.), Bacterial stress responses. American Society for Microbiology, Washington, D.C.

47. **Futai, E., T. Kubo, H. Sorimachi, K. Suzuki, and T. Maeda.** 2001. Molecular cloning of PalBH, a mammalian homologue of the *Aspergillus* atypical calpain PalB. Biochim. Biophys. Acta **1517:**316–319.

48. **Futai, E., T. Maeda, H. Sorimachi, K. Kitamoto, S. Ishiura, and K. Suzuki.** 1999. The protease activity of a calpain-like cysteine protease in *Saccharomyces cerevisiae* is required for alkaline adaptation and sporulation. Mol. Gen. Genet. **260:**559–568.

49. **Gale, E. F.** 1946. The bacterial amino acid decarboxylases. Adv. Enzymol. **6:**1–32.

50. **Gale, E. F., and H. M. R. Epps.** 1942. The effect of the *p*H of the medium during growth on enzymic activities of bacteria (*Escherichia coli* and *Micrococcus lysodeikticus*) and the biological significance of the changes produced. Biochem. J. **36:**600–618.

51. **Gancedo, J. M.** 2001. Control of pseudohyphae formation in *Saccharomyces cerevisiae*. FEMS Microbiol. Rev. **25:**107–123.

52. **Gavin, A. C., M. Bosche, R. Krause, P. Grandi, M. Marzioch, A. Bauer, J. Schultz, J. M. Rick, A. M. Michon, C. M. Cruciat, M. Remor, C. Hofert, M. Schelder, M. Brajenovic, H. Ruffner, A. Merino, K. Klein, M. Hudak, D. Dickson, T. Rudi, V. Gnau, A. Bauch, S. Bastuck, B. Huhse, C. Leutwein, M. A. Heurtier, R. R. Copley, A. Edelmann, E. Querfurth, V. Rybin, G. Drewes, M. Raida, T. Bouwmeester, P. Bork, B. Seraphin, B. Kuster, G. Neubauer, and G. Superti-Furga.** 2002. Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature **415:**141–147.

53. **Ghannoum, M. A., B. Spellberg, S. M. Saporito-Irwin, and W. A. Fonzi.** 1995. Reduced virulence in *Candida albicans PHR1* mutants. Infect. Immun. **63:**4528–4530.

54. **Gielkens, M., L. González-Candelas, P. Sánchez-Torres, P. van de Vondervoort, L. de Graaff, J. Visser, and D. Ramón.** 1999. The *abfB* gene encoding the major α-L-arabinofuranosidase of *Aspergillus nidulans*: nucleotide sequence, regulation and construction of a disrupted strain. Microbiology **145:**735–741.

55. **Gimeno, C. J., and G. R. Fink.** 1994. Induction of pseudohyphal growth by overexpression of *PHD1*, a *Saccharomyces cerevisiae* gene related to transcriptional regulators of fungal development. Mol. Cell. Biol. **14:**2100–2112.

56. **González-López, C. I., R. Szabo, S. Blanchin-Roland, and C. Gaillardin.** 2002. Genetic control of extracellular protease synthesis in the yeast *Yarrowia lipolytica*. Genetics **160:**417–427.

57. **Hall, H. K., K. L. Karem, and J. W. Foster.** 1995. Molecular responses of microbes to environmental pH stress. Adv. Microb. Physiol. **37:**229–272.

58. **Heinz, W. J., O. Kurzai, A. A. Brakhage, W. A. Fonzi, H. C. Korting, M. Frosch, and F. A. Mühlschlegel.** 2000. Molecular responses to changes in the environmental pH are conserved between the fungal pathogens *Candida dubliniensis* and *Candida albicans*. Int. J. Med. Microbiol. **290:**231–238.

59. **Hutchings, H., K.-P. Stahmann, S. Roels, E. A. Espeso, W. E. Timberlake, H. N. Arst, Jr., and J. Tilburn.** 1999. The multiply-regulated *gabA* gene encoding the GABA permease of *Aspergillus nidulans*: a score of exons. Mol. Microbiol. **32:**557–568.

60. **Ito, T., T. Chiba, R. Ozawa, M. Yoshida, M. Hattori, and Y. Sakaki.** 2001. A comprehensive two-hybrid analysis to explore the yeast protein interactome. Proc. Natl. Acad. Sci. USA **98:**4569–4574.

61. **Jarai, G., and F. Buxton.** 1994. Nitrogen, carbon, and pH regulation of extracellular acidic proteases of *Aspergillus niger*. Curr. Genet. **26:**238–244.

62. **Katz, M. E., R. N. Rice, and B. F. Cheetham.** 1994. Isolation and characterization of an *Aspergillus nidulans* gene encoding an alkaline protease. Gene **150:**287–292.

63. **Katzmann, D. J., M. Babst, and S. D. Emr.** 2001. Ubiquitin-dependent sorting into the multivesicular body pathway requires the function of a conserved endosomal protein sorting complex, ESCRT-I. Cell **106:**145–155.

64. **Keller, N. P., C. Nesbitt, B. Sarr, T. D. Phillips, and G. B. Burow.** 1997. pH regulation of sterigmatocystin and aflatoxin biosynthesis in *Aspergillus* spp. Phytopathology **87:**643–648.

65. **Kobayashi, H., H. Sito, and T. Kakegawa.** 2000. Bacterial strategies to inhabit acidic environments. J. Gen. Appl. Microbiol. **46:**235–243.

66. **Krebs, J., and R. Klemenz.** 2000. The ALG-2/AIP-complex, a modulator at the interface between cell proliferation and cell death? A hypothesis. Biochim. Biophys. Acta **1498:**153–161.

67. **Lacadena, J., A. Martínez del Pozo, M. Gasset, B. Patino, R. Campos-Olivas, C. Vázquez, A. Martinez-Ruiz, J. M. Mancheno, M. Onaderra, and J. G. Gavilanes.** 1995. Characterization of the antifungal protein secreted by the mould *Aspergillus giganteus*. Arch. Biochem. Biophys. **324:**273–281.

68. **Lamb, T. M., W. Xu, A. Diamond, and A. P. Mitchell.** 2001. Alkaline response genes of *Saccharomyces cerevisiae* and their relationship to the *RIM101* pathway. J. Biol. Chem. **276:**1850–1856.

69. **Lambert, M., S. Blanchin-Roland, F. Le Louedec, A. Lépingle, and C. Gaillardin.** 1997. Genetic analysis of regulatory mutants affecting synthesis of extracellular proteinases in the yeast *Yarrowia lipolytica*: identification of a *RIM101/pacC* homolog. Mol. Cell. Biol. **17:**3966–3976.

70. **Lengeler, K. B., R. C. Davidson, C. D'souza, T. Harashima, W.-C. Shen, P. Wang, X. Pan, M. Waugh, and J. Heitman.** 2000. Signal transduction cascades regulating fungal development and virulence. Microbiol. Mol. Biol. Rev. **64:**746–785.

71. **Li, W. S., and A. P. Mitchell.** 1997. Proteolytic activation of Rim1p, a positive regulator of yeast sporulation and invasive growth. Genetics **145:**63–73.

72. **Lindberg, R. A., W. G. Rhodes, L. D. Eirich, and H. Drucker.** 1982. Extracellular acid proteases from *Neurospora crassa*. J. Bacteriol. **150:**1103–1108.

72a.**Liu, H.** 2001. Transcriptional control of dimorphism in *Candida albicans*. Curr. Opin. Microbiol. **4:**728–735.

73. **Lo, H.-J., J. R. Köhler, B. Di Domenico, D. Loebenberg, A. Cacciapuoti, and G. R. Fink.** 1997. Nonfilamentous *C. albicans* mutants are avirulent. Cell **90:**939–949.

74. **Lo, K. W., Q. Zhang, M. Li, and M. Zhang.** 1999. Apoptosis-linked gene product ALG-2 is a new member of the calpain small subunit subfamily of Ca^{2+}-binding proteins. Biochemistry **38:**7498–7508.

75. **MacCabe, A. P., M. Orejas, J. A. Pérez-González, and D. Ramón.** 1998. Opposite patterns of expression of two *Aspergillus nidulans* xylanase genes with respect to ambient pH. J. Bacteriol. **180:**1331–1333.

76. **MacCabe, A. P., J. P. T. W. van den Hombergh, J. Tilburn, H. N. Arst, Jr., and J. Visser.** 1996. Identification, cloning and analysis of the *Aspergillus niger* gene *pacC*, a wide domain regulatory gene responsive to ambient pH. Mol. Gen. Genet. **250:**367–374.

77. **Maccheroni, W., G. S. May, N. M. Martinez-Rossi, and A. Rossi.** 1997. The sequence of *palF*, an environmental pH response gene in *Aspergillus nidulans*. Gene **194:**163–167.

78. **Madzak, C., S. Blanchin-Roland, O. R. Cordero, and C. Gaillardin.** 1999. Functional analysis of upstream regulating regions from the *Yarrowia lipolytica XPR2* promoter. Microbiology **145:**75–87.

79. **McGrath, J. W., and J. P. Quinn.** 2000. Intracellular accumulation of polyphosphate by the yeast *Candida humicola* G-1 in response to acid pH. Appl. Environ. Microbiol. **66:**4068–4073.

80. **Meyer, V., M. Wedde, and U. Stahl.** 2002. Transcriptional regulation of the antifungal protein in *Aspergillus giganteus*. Mol. Genet. Genomics **266:**747–757.

81. **Mingot, J. M.** 1999. Procesamiento proteolítico y regulación de la localización subcelular del factor de transcripción PacC por el pH ambiental. Ph.D. thesis. Facultad de Farmacia, Universidad Complutense de Madrid, Madrid, Spain.

82. **Mingot, J. M., E. A. Espeso, E. Díez, and M. A. Peñalva.** 2001. Ambient pH signaling regulates nuclear localization of the *Aspergillus nidulans* PacC transcription factor. Mol. Cell. Biol. **21:**1688–1699.

83. **Mingot, J. M., J. Tilburn, E. Díez, E. Bignell, M. Orejas, D. A. Widdick, S. Sarkar, C. V. Brown, M. X. Caddick, E. A. Espeso, H. N. Arst, Jr., and M. A. Peñalva.** 1999. Specificity determinants of proteolytic processing of *Aspergillus* PacC transcription factor are remote from the processing site, and processing occurs in yeast if pH signalling is bypassed. Mol. Cell. Biol. **19:**1390–1400.

84. **Missotten, M., A. Nichols, K. Rieger, and R. Sadoul.** 1999. Alix, a novel mouse protein undergoing calcium-dependent interaction with the apoptosis-linked-gene 2 (ALG-2) protein. Cell Death Differ. **6:**124–129.

85. **Mitchell, A. P.** 1998. Dimorphism and virulence in *Candida albicans*. Curr. Opin. Microbiol. **1:**687–692.

86. **Moretti, M. B., A. Batlle, and S. C. Garcia.** 2001. *UGA4* gene encoding the γ-aminobutyric acid permease in *Saccharomyces cerevisiae* is an acid-expressed gene. Int. J. Biochem. Cell Biol. **33:**1202–1207.

87. **Mouyna, I., T. Fontaine, M. Vai, M. Monod, W. A. Fonzi, M. Diaquin, L. Popolo, R. P. Hartland, and J. P. Latgé.** 2001. Glycosylphosphatidylinositol-anchored glucanosyltransferases play an active role in the biosynthesis of the fungal cell wall. J. Biol. Chem. **275:**14882–14889.

88. **Mühlschlegel, F. A., and W. A. Fonzi.** 1997. *PHR2* of *Candida albicans* encodes a functional homolog of the pH-regulated gene *PHR1* with an inverted pattern of pH-dependent expression. Mol. Cell. Biol. **17:**5960–5967.

89. **Nahas, E., H. F. Terenzi, and A. Rossi.** 1982. Effect of carbon source and pH on the production and secretion of acid phosphatase (EC 3.1.3.2) and alkaline phosphatase (EC 3.1.3.1) in *Neurospora crassa*. J. Gen. Microbiol. **128:**2017–2021.

90. **Negrete-Urtasun, S., S. H. Denison, and H. N. Arst, Jr.** 1997. Characterization of the pH signal transduction pathway gene *palA* of *Aspergillus nidulans* and identification of possible homologs. J. Bacteriol. **179:**1832–1835.

91. **Negrete-Urtasun, S., W. Reiter, E. Díez, S. H. Denison, J. Tilburn, E. A. Espeso, M. A. Peñalva, and H. N. Arst, Jr.** 1999. Ambient pH signal transduction in *Aspergillus*: completion of gene characterization. Mol. Microbiol. **33:**994–1003.

92. **Nickas, M. E., and M. P. Yaffe.** 1996. *BRO1*, a novel gene that interacts with components of the Pkc1p-mitogen-activated-protein kinase pathway in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **16:**2585–2593.

93. **Nozawa, S. R., and A. Rossi.** 2000. Gene *pac*A⁺ codes for the multiple active forms of P_i-repressible acid phosphatase in the mould *Aspergillus nidulans*. World J. Microbiol. Biotechnol. **16:**333–336.

94. **Ogawa, N., J. DeRisi, and P. O. Brown.** 2000. New components of a system for phosphate accumulation and polyphosphate metabolism in *Saccharomyces cerevisiae* revealed by genomic expression analysis. Mol. Biol. Cell **11:**4309–4321.

95. **Orejas, M., E. A. Espeso, J. Tilburn, S. Sarkar, H. N. Arst, Jr., and M. A. Peñalva.** 1995. Activation of the *Aspergillus* PacC transcription factor in response to alkaline ambient pH requires proteolysis of the carboxy-terminal moiety. Genes Dev. **9:**1622–1632.

96. **Orejas, M., A. P. MacCabe, J. A. Pérez-Gonzalez, S. Kumar, and D. Ramón.** 1999. Carbon catabolite repression of the *Aspergillus nidulans xlnA* gene. Mol. Microbiol. **31:**177–184.

97. **Peñalva, M. A., R. T. Rowlands, and G. Turner.** 1998. The optimization of penicillin biosynthesis in fungi. Trends Biotechnol. **16:**483–489.

98. **Pérez-González, J. A., L. H. de Graaff, J. Visser, and D. Ramón.** 1996. Molecular cloning and expression in *Saccharomyces cerevisiae* of two *Aspergillus nidulans* xylanase genes. Appl. Environ. Microbiol. **62:**2179–2182.

99. **Porta, A., A. M. Ramón, and W. A. Fonzi.** 1999. *PRR1*, a homolog of *Aspergillus nidulans palF*, controls pH-dependent gene expression and filamentation in *Candida albicans*. J. Bacteriol. **181:**7516–7523.

100. **Porta, A., Z. Wang, A. Ramón, F. A. Muhlschlegel, and W. A. Fonzi.** 2002. Spontaneous second-site suppressors of the filamentation defect of *prr1Δ* mutants define a critical domain of Rim101p in *Candida albicans*. Mol. Genet. Genomics **266:**624–631.

101. **Poussereau, N., S. Creton, G. Billon-Grand, C. Rascle, and M. Fevre.** 2001. Regulation of *acp1*, encoding a non-aspartyl acid protease expressed during pathogenesis of *Sclerotinia sclerotiorum*. Microbiology **147:**717–726.

102. **Prusky, D., J. L. McEvoy, B. Leverentz, and W. S. Conway.** 2001. Local modulation of host pH by *Colletotrichum* species as a mechanism to increase virulence. Mol. Plant-Microbe Interact. **14:**1105–1113.

103. **Purnell, D. M., and G. M. Martin.** 1971. *Aspergillus nidulans*: association of certain alkaline phosphatase mutations with decreased virulence for mice. J. Infect. Dis. **123:**305–306.

104. **Ramón, A. M., A. Porta, and W. A. Fonzi.** 1999. Effect of environmental pH on morphological development of *Candida albicans* is mediated via the

PacC-related transcription factor encoded by *PRR2*. J. Bacteriol. **181:**7524–7530.

105. **Roberts, R. L., and G. R. Fink.** 1994. Elements of a single MAP kinase cascade in *Saccharomyces cerevisiae* mediate two developmental programs in the same cell type: mating and invasive growth. Genes Dev. **8:**2974–2985.

106. **Rollins, J. A., and M. B. Dickman.** 2001. pH signaling in *Sclerotinia sclerotiorum*: identification of a *pacC/RIM1* homolog. Appl. Environ. Microbiol. **67:**75–81.

107. **Sakurai, Y., and H. Shiota.** 1977. Two distinct types of acid phosphatase formation in *Aspergillus* sp. J. Gen. Appl. Microbiol. **23:**311–323.

108. **Saporito-Irwin, S. M., C. E. Birse, P. S. Sypherd, and W. A. Fonzi.** 1995. *PHR1*, a pH-regulated gene of *Candida albicans*, is required for morphogenesis. Mol. Cell. Biol. **15:**601–613.

109. **Sarkar, S., M. X. Caddick, E. Bignell, J. Tilburn, and H. N. Arst, Jr.** 1996. Regulation of gene expression by ambient pH in *Aspergillus*: genes expressed at acid pH. Biochem. Soc. Trans. **24:**360–363.

110. **Schmitt, E. K., R. Kempken, and U. Kuck.** 2001. Functional analysis of promoter sequences of cephalosporin C biosynthesis genes from *Acremonium chrysogenum*: specific DNA-protein interactions and characterization of the transcription factor PACC. Mol. Genet. Genomics **265:**508–518.

111. **Sentandreu, M., M. V. Elorza, R. Sentandreu, and W. A. Fonzi.** 1998. Cloning and characterization of *PRA1*, a gene encoding a novel pH-regulated antigen of *Candida albicans*. J. Bacteriol. **180:**282–289.

112. **Shah, A. J., J. Tilburn, M. W. Adlard, and H. N. Arst, Jr.** 1991. pH regulation of penicillin production in *Aspergillus nidulans*. FEMS Microbiol. Lett. **77:**209–212.

113. **Sorimachi, H., S. Ishiura, and K. Suzuki.** 1997. Structure and physiological functions of calpains. Biochem. J. **328:**721–732.

114. **Sorimachi, H., and K. Suzuki.** 2001. The structure of calpain. J. Biochem. **129:**653–664.

115. **St. Leger, R. J., J. O. Nelson, and S. E. Screen.** 1999. The entomopathogenic fungus *Metarhizium anisopliae* alters ambient pH, allowing extracellular protease production and activity. Microbiology **145:**2691–2699.

116. **Stoldt, V. R., A. Sonneborn, C. E. Leuker, and J. F. Ernst.** 1997. Efg1p, an essential regulator of morphogenesis of the human pathogen *Candida albicans*, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi. EMBO J. **16:**1982–1991.

117. **Su, S. S., and A. P. Mitchell.** 1993. Identification of functionally related genes that stimulate early meiotic gene expression in yeast. Genetics **133:**67–77.

118. **Su, S. S., and A. P. Mitchell.** 1993. Molecular characterization of the yeast meiotic regulatory gene *RIM1*. Nucleic Acids Res. **21:**3789–3797.

119. **Suárez, T., and M. A. Peñalva.** 1996. Characterization of a *Penicillium chrysogenum* gene encoding a PacC transcription factor and its binding sites in the divergent *pcbAB-pcbC* promoter of the penicillin biosynthetic cluster. Mol. Microbiol. **20:**529–540.

120. **Szabo, R., and V. Stofanikova.** 2002. Presence of organic sources of nitrogen is critical for filament formation and pH-dependent morphogenesis in *Yarrowia lipolytica*. FEMS Microbiol. Lett. **206:**45–50.

121. **Then-Bergh K., and A. A. Brakhage.** 1998. Regulation of the *Aspergillus nidulans* penicillin biosynthesis gene *acvA* (*pcbAB*) by amino acids: implication for involvement of transcription factor PACC. Appl. Environ. Microbiol. **64:**843–849.

122. **Tilburn, J., S. Sarkar, D. A. Widdick, E. A. Espeso, M. Orejas, J. Mungroo, M. A. Peñalva, and H. N. Arst, Jr.** 1995. The *Aspergillus* PacC zinc finger transcription factor mediates regulation of both acid- and alkaline-expressed genes by ambient pH. EMBO J. **14:**779–790.

123. **Toh-e, A., S. Kobayashi, and Y. Oshima.** 1978. Disturbance of the machinery for the gene expression by acidic pH in the repressible acid phosphatase system of *Saccharomyces cerevisiae*. Mol. Gen. Genet. **162:**139–149.

124. **Tréton, B., S. Blanchin-Roland, M. Lambert, A. Lepingle, and C. Gaillardin.** 2000. Ambient pH signalling in ascomycetous yeasts involves homologues of the *Aspergillus nidulans* genes *palF* and *palH*. Mol. Gen. Genet. **263:**505–513.

125. **Uetz, P., L. Giot, G. Cagney, T. A. Mansfield, R. S. Judson, J. R. Knight, D. Lockshon, V. Narayan, M. Srinivasan, P. Pochart, A. Qureshi-Emili, Y. Li, B. Godwin, D. Conover, T. Kalbfleisch, G. Vijayadamodar, M. Yang, M. Johnston, S. Fields, and J. M. Rothberg.** 2000. A comprehensive analysis of protein-protein interactions in *Saccharomyces cerevisiae*. Nature **403:**623–627.

126. **van den Hombergh, J. P. T. W., G. Jarai, F. P. Buxton, and J. Visser.** 1994. Cloning, characterization and expression of *pepF*, a gene encoding a serine carboxypeptidase from *Aspergillus niger*. Gene **151:**73–79.

127. **van den Hombergh, J. P. T. W., A. P. MacCabe, P. J. I. van de Vondervoort, and J. Visser.** 1996. Regulation of acid phosphatases in an *Aspergillus niger pacC* disruption strain. Mol. Gen. Genet. **251:**542–550.

128. **van Kuyk, P. A., B. F. Cheetham, and M. E. Katz.** 2000. Analysis of two *Aspergillus nidulans* genes encoding extracellular proteases. Fungal Genet. Biol. **29:**201–210.

129. **van Peij, N. N., M. M. Gielkens, R. P. de Vries, J. Visser, and L. H. de Graaff.** 1998. The transcriptional activator XlnR regulates both xylanolytic and endoglucanase gene expression in *Aspergillus niger*. Appl. Environ. Microbiol. **64:**3615–3619.

130. **Vito, P., L. Pellegrini, C. Guiet, and L. D'Adamio.** 1999. Cloning of AIP1, a novel protein that associates with the apoptosis-linked gene ALG-2 in a Ca^{2+}-dependent reaction. J. Biol. Chem. **274:**1533–1540.

131. **Wallis, G. L., R. J. Swift, R. Atterbury, S. Trappe, U. Rinas, F. W. Hemming, M. G. Wiebe, A. P. Trinci, and J. F. Peberdy.** 2001. The effect of pH on glucoamylase production, glycosylation and chemostat evolution of *Aspergillus niger*. Biochim. Biophys. Acta **1527:**112–122.

132. **Wiebe, M. G., G. D. Robson, J. R. Shuster, and A. P. Trinci.** 1999. pH regulation of recombinant glucoamylase production in *Fusarium venenatum* JeRS 325, a transformant with a *Fusarium oxysporum* alkaline (trypsin-like) protease promoter. Biotechnol. Bioeng. **64:**368–372.

133. **Wilson, R. B., D. Davis, and A. P. Mitchell.** 1999. Rapid hypothesis testing with *Candida albicans* through gene disruption with short homology regions. J. Bacteriol. **181:**1868–1874.

133a.**Wu, Y., S. Pan, S. Che, G. He, M. Nelman-Gonzalez, M. M. Weil, and J. Kuang.** 2001. Overexpression of Hp95 induces G1 phase arrest in confluent HeLa cells. Differentiation **67:**139–153.

134. **Wurst, H., T. Shiba, and A. Kornberg.** 1995. The gene for a major exopolyphosphatase of *Saccharomyces cerevisiae*. J. Bacteriol. **177:**898–906.

135. **Xu, W., and A. P. Mitchell.** 2001. Yeast PalA/AIP1/Alix homolog Rim20p associates with a PEST-like region and is required for its proteolytic cleavage. J. Bacteriol. **183:**6917–6923.

136. **Yakoby, N., I. Kobiler, A. Dinoor, and D. Prusky.** 2000. pH regulation of pectate lyase secretion modulates the attack of *Colletotrichum gloeosporioides* on avocado fruits. Appl. Environ. Microbiol. **66:**1026–1030.

137. **Yaver, D. S., M. Lamsa, R. Munds, S. H. Brown, S. Otani, L. Franssen, J. A. Johnstone, and H. Brody.** 2000. Using DNA-tagged mutagenesis to improve heterologous protein production in *Aspergillus oryzae*. Fungal Genet. Biol. **29:**28–37.