THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 46, Issue of November 15, pp. 28825–28830, 1996

Printed in U.S.A.

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*

(Received for publication, July 8, 1996, and in revised form, August 5, 1996)

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The isopenicillin N synthase (*ipnA*) gene, encoding a key penicillin biosynthetic enzyme in *Aspergillus nidulans*, represents a prototype of an alkaline-expressed gene. *ipnA* is under ambient pH regulation, and its promoter (*ipnA*^{P}) contains binding sites for the zinc-finger transcription factor PacC. We show here that three of these sites, denoted ipnA2, ipnA3, and ipnA4AB, are efficiently recognized by the protein in an isolated sequence context. Single, double, and triple inactivation of these sites in any possible combination reduced promoter activity under alkaline conditions but had no effect under acidic conditions (under which promoter activity was low), as measured by the expression of wild-type and mutant *ipnA*^{P}::*lacZ* fusion genes integrated in single copy into a common chromosomal location. This establishes a physiological role for these PacC binding sites and demonstrates a direct role for PacC in ambient pH regulation of *ipnA* gene expression. In addition, this confirms our previous proposal that PacC is an activator for alkaline-expressed genes. Notably, our experiments show that ipnA2, the highest affinity site for PacC in the *ipnA*^{P}, contributes relatively modestly to PacC-mediated activation. By contrast, the lower affinity sites ipnA3 and ipnA4AB contribute more substantially to regulation by ambient pH. Inactivation of these three binding sites reduced promoter activity under alkaline conditions to that observed under acidic conditions, showing that these three PacC sites at *ipnA*^{P} are sufficient to account for its activation by alkaline ambient pH.

Saprophytic microbes depend largely upon secretion of extracellular enzymes and small bioactive metabolites for survival in nature, and these molecules, acting beyond the permeability barrier, are exposed to variations in ambient pH. In common with many of these microorganisms, the filamentous ascomycete *Aspergillus nidulans* is able to grow over a wide pH range (1), adapting the pattern of expression of the genes involved in the synthesis of the above molecules to the necessities imposed by ambient pH. pH regulation of gene expression ensures, for example, the synthesis of acid phosphatase predominantly at acidic ambient pH values at which this secreted enzyme shows optimal activity.

Since the pioneering description of pH regulation and its genetic analysis in *A. nidulans* by Caddick *et al.* (2), this regulatory circuit has been thoroughly analyzed both genetically and molecularly. In the current model, alkaline pH is registered by a signal transduction pathway involving the products of at least six *pal* genes (3, 4) and transmitted to a transcription factor encoded by the *pacC* regulatory gene (5), which thereby switches from an inactive to an active form. This active PacC protein is able to activate transcription of alkaline-expressed genes and repress transcription of acid-expressed genes. The absence of the alkaline pH signal therefore prevents activation of PacC, resulting in derepression of acidic genes and lack of activation of alkaline genes (5, 6). Genes encoding PacC isofunctional homologues have been described in other filamentous fungi, such as *Aspergillus niger* (7) and *Penicillium chrysogenum* (8).

The PacC version that is inactive in structural gene regulation is the 678-residue primary translation product. This is converted into an active version by proteolytic removal of ~60% of the residues of the protein at the carboxyl side (6). The resulting polypeptide, consisting of approximately residues 1–270 and including the DNA binding domain, activates transcription of "alkaline" genes and represses transcription of "acidic" genes.

The DNA binding domain of PacC consists of three zinc fingers of the Cys_2His_2 type, specifically recognizing a core consensus hexanucleotide of sequence 5′-GCCARG-3′ (5). The presence of adenosine in position 4 of this hexanucleotide is essential for its binding by either the *A. nidulans* (5) or the *P. chrysogenum* (8) PacC zinc-finger region. The *ipnA*[1] (isopenicillin N synthase gene), encoding a key enzyme in penicillin biosynthesis, is a prototype of an alkaline-expressed gene (9). Its promoter (*ipnA*^{P}) contains four *in vitro* binding sites for a GST:PacC fusion protein containing the PacC DNA binding domain (5, 6), and we have therefore proposed that PacC directly mediates elevated expression of *ipnA* under alkaline conditions. Here, we use targeted mutations in each of the PacC binding sites in an *ipnA*^{P}::*lacZ* fusion (10) to establish that these sites are essential *in vivo* for PacC-dependent activation of *ipnA*, confirming their physiological relevance and providing definitive evidence that PacC is a transcriptional activator for alkaline-expressed genes. Remarkably, the highest affinity site for PacC makes a smaller contribution to promoter activation than other, lower affinity sites.

EXPERIMENTAL PROCEDURES

A. nidulans Strains and Media—*A. nidulans* strains carried markers in standard use (11). *pacC*^{c}14 is an alkalinity-mimicking gain-of-func-

* This work was supported by grants from the Comisión Interministerial de Ciencia y Tecnología, Spain (BIO94-0932) and the European Commission (BIO-CT93-0174) (to M. A. P.) The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: *ipnA*, isopenicillin N synthase gene; *ipnA*^{P}, *ipnA* promoter; GST, glutathione thiotransferase; PCR, polymerase chain reaction; bp, base pair(s).

TABLE I
Oligonucleotides used in this work

Oligonucleotide	Sequence	Coordinates[a]
CREA-B	5′-GATCGACGCAGCAACAGTATGTGAGCCACC-3′	−1345 to −1316
PACC-A	5′-CGACTGGAATCGGCCAG-3′	−707 to −723
PACC-B	5′-ACAAGATTAGGGACTGACTTCG-3′	−685 to −664
IPNA3*[b]	5′-GTGACGGTGTCGCTAGGCCGTC-3′	−591 to −612
IPNA4AB*[b]	5′-ACTGCAGCAATAGCCCCGCCTGGAATAGAGGGCTAGGCT-3′	−327 to −365

[a] Coordinates given as distance to the *ipnA* translation initiation codon.
[b] Mutated nucleotides are *underlined.*

tion mutation in *pacC* (5). Standard media (2, 12) were used for culturing strains and obtaining conidiospores. Cultures for β-galactosidase assays were inoculated as described (10). Alkaline or acidic growth conditions were achieved using penicillin production broth (13) containing 3% (w/v) sucrose and 100 mM HEPES. For alkaline cultures (final pH, ~7.6), initial pH was adjusted to 8.0 with NaOH. For acidic conditions, no NaOH was added, resulting in an initial pH value of 6.8. HEPES showed no buffering capacity at this pH value, and sucrose utilization by the fungus resulted in substantial acidification of this medium (final pH, 5.6). The extra Na^+ ions introduced as NaOH in alkaline broth were included in acidic broth as NaCl. Mycelia were harvested after 18 or 24 h of growth at 37 °C and used for protein extraction.

Transformation of A. nidulans with lacZ Constructs—A. nidulans was transformed by standard procedures (14). The recipient strain for transformation with *ipnA*^{p}::*lacZ* constructs was WG355 (*biA*1, *argB*2, and *bgA*0). Homokaryotic *argB*$^+$-transformed clones were purified, and the presence of a single-copy integration event of the transforming plasmid at either the *ipnA* or *argB* locus was analyzed by Southern blot hybridization using appropriate probes (10). Two independent transformants were isolated for each construct and integration event. In all cases, both transformants were shown to have the same phenotype with regard to *lacZ* expression. Integration of the *ipnA*^{p}(-2)::*lacZ* construct (deleted for the PacC ipnA2 binding site; see "Results" for nomenclature) at the *ipnA* locus by a single recombination step involving sequences upstream or downstream of the chromosomal PacC ipnA2 site would result in placing the transforming *lacZ* gene or the resident *ipnA* gene, respectively, downstream of the mutated promoter. Transformants of the former type, in which the mutated promoter drives expression of the *lacZ* fusion, were distinguished by Southern blot hybridization because the promoter deleted for the ipnA2 site lacks a diagnostic *Stu*I site that is present in the wild-type promoter.

Standard parasexual genetics were used to construct strains carrying the *ipnA*^{p}::*lacZ* or the *ipnA*^{p}(-2)::*lacZ* fusion in a *pacC*^{c} background. Diploids were constructed using recombinant strains carrying either of these *lacZ* fusions and a strain of genotype *yA*2, *pabaA*1, *argB*2, and *pacC*^{c}*14*. Haploid *argB*$^+$ segregants (thereby carrying the transforming construct) were screened for the presence of the *pacC*^{c}14 mutation by means of the hypersensitivity to neomycin and reduced conidiation phenotypes resulting from this mutation (5). Two independent segregants were selected for each construct. The presence in single copy of the *lacZ* fusion genes at the *argB* locus of these segregants in all cases was confirmed by Southern analysis.

β-Galactosidase Assays—Protein extraction (from lyophilized mycelia) and β-galactosidase assays were as described (10). Activities were calculated as the average value obtained from at least three independent cultures for each transformant.

Electrophoretic Mobility Shift Assays—Purification of a bacterially expressed GST::PacC(30–195*) fusion protein carrying the PacC zinc-finger DNA binding domain and preparation of double-stranded oligonucleotides carrying PacC binding sites (see "Results" for nucleotide sequences) have been described (5). Binding reactions were carried out in the presence of 15 fmol of labeled double-stranded oligonucleotide and the indicated amounts of fusion protein essentially as described (5, 15). Protein-DNA complexes were resolved in 4% polyacrylamide gels run in 0.5 × Tris borate EDTA. Gels were dried and exposed to a PhosphorImager screen for quantitative analysis of the radioactivity present in each band.

Construction of ipnA^p::lacZ Fusion Genes with Mutations in the PacC Binding Sites—Plasmid pBSΔ1 (10) is a pBS-SK+ derivative carrying an *ipnA*^{p}::*lacZ* fusion and an *argB*$^+$ transformation marker. Mutant promoter fragments were used to substitute wild-type sequences in this construct. A promoter fragment carrying a deletion of the ipnA2 PacC binding site was constructed as follows: plasmid pBS-Bam (10), which contains a 2-kilobase pair fragment of the *ipnA* upstream region, was used as a template in two separate PCR amplifications using primer pairs CREA-B/PACC-A and PACC-B/Universal primer (Pharmacia Biotech Inc.), respectively (for these and other oligonucleotide primers, see Table I). The two resulting fragments were blunted with T4 DNA polymerase and digested with *Bst*EII or *Bss*HII, respectively. Ligation of these fragments into pBS-Bam double-digested with these two enzymes reconstructed an *ipnA*^{p} fragment from which a 21-bp sequence including site ipnA2 had been precisely deleted (plasmid pBS-Bam[Δipn2]). An *ipnA* promoter carrying a site-destroying $A_4 \rightarrow T$ mutation in ipnA3 was constructed as follows: oligonucleotides IPNA3* and CREA-B were used to PCR-amplify a 748-bp promoter fragment carrying the said point mutation. A mutant 159-bp *Sac*I-*Asp*I subfragment of this amplification product was used to substitute for the corresponding wild-type fragment (plasmid pBS-Bam[ipnA3*]). A promoter with a double $A_4 \rightarrow T$ site-destroying mutation in site ipnA4AB was reconstructed in a similar way after PCR amplification with primers CREA-B and IPNA4AB*. A 112-bp mutant *Hin*dIII-*Pst*I fragment included in this amplification product was used in a two-step procedure in which a 281-bp *Hin*dIII-*Bss*HII fragment carrying the mutated ipn4AB site was used to replace wild-type sequences in plasmid pBS-Bam (plasmid pBS-Bam[ipnA4AB*]). A promoter carrying mutations in both ipnA2 and ipnA3 (plasmid pBS-Bam[Δipn2, ipnA3*]) was constructed as the single ipnA3 mutant, using plasmid pBS-Bam[Δipn2] as a template for PCR amplification. To construct double mutant promoters in sites ipnA2 and ipnA4AB and sites ipnA3 and ipnA4AB and the triple mutant promoter in sites ipnA2, ipnA3, and ipnA4AB, the 281-bp *Hin*dIII-*BssH*II wild-type fragment from plasmids pBS-Bam[Δipn2], pBS-Bam[ipnA3*], and pBS-Bam[Δipn2, ipnA3*] was replaced by the mutant fragment carrying site-destroying mutations in site ipnA4AB to yield plasmids pBS-Bam[Δipn2, ipnA4AB*], pBS-Bam[ipnA3*, ipnA4AB*], and pBS-Bam[Δipn2, ipnA3*, ipnA4AB*], respectively. In all cases, the regions obtained by PCR amplification were fully sequenced to rule out the presence of additional mutations introduced during the amplification procedure.

RESULTS

Relative Affinity of the PacC Zinc-finger Region for its Binding Sites at the ipnA Promoter—We have previously determined using DNase I footprinting analysis the presence in the *ipnA* promoter of three single and one double binding sites for a bacterially expressed GST::PacC(30–195*) fusion protein (5), whose relative positions to the *ipnA* major transcription start point are shown in Fig. 1. For a preliminary comparison of their relative affinities, double-stranded oligonucleotides including the region protected from DNase I digestion, each containing just one of the above binding sites, were synthesized (Fig. 2*A*) and tested for binding of the purified protein.

Single sites ipnA1, ipnA2, and ipnA3 were all contained in a *Sac*I-*Spe*I *ipnA* promoter fragment (−654 to −459 relative to the major transcription start point (10)). Strong binding was detected with the ipnA2 site (Fig. 2*B*), even at the lowest protein concentration tested and consistent with previous data indicating that ipnA2 is the highest affinity site (5). By contrast, no binding was detected with site ipnA1 (Fig. 2*B*) even after long exposures using a large excess (0.5 μg) of the fusion protein, strongly indicating that significant binding to this site occurs only in the presence of the neighboring ipnA2 and ipnA3 sites. Binding to ipnA3 (Fig. 2*B*) or ipnA4AB (data not shown) was weaker than that detected for ipnA2.

To more precisely quantify the relative affinity of PacC for each of the binding sites, we used competition experiments in

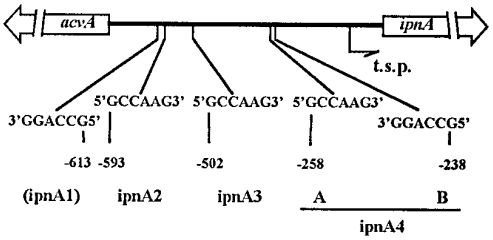

FIG. 1. **Position of the *in vitro* PacC binding sites in the *ipnA* promoter.** The *ipnA* gene is transcribed divergently from *acvA*, encoding the first enzyme of the penicillin pathway. The scheme shows the location of these binding sites in the *ipnA* upstream region, as determined by Tilburn *et al.* (5). All these sites map to the 872-bp intergenic *acvA-ipnA* region. The precise position of these sites is given as the distance from the first guanosine of the core consensus PacC hexanucleotide to the major transcription start point for *ipnA*, located 106 bp upstream from its translation start codon (10). The sequence of the *acvA-ipnA* intergenic region was taken from Ref. 19. As shown here, site ipnA1 (shown in *parentheses*) has a negligible affinity for PacC in an isolated context, and it was therefore not considered further. It was almost certainly detected in previous experiments due to the dimeric nature of the GST::PacC fusion protein in association with the close proximity of the high affinity binding site ipnA2. Site ipnA4 is a dual site containing two inverted repeats of the 5′-GCCARG-3′ core hexanucleotide separated by 9 bp.

which binding of the fusion protein to the ^{32}P-labeled highest affinity site ipnA2 was competed with different amounts of each of the unlabeled synthetic sites. Fig. 2*C* shows that the amount of cold double-stranded oligonucleotide needed for a 50% reduction in the protein-ipnA2 complex formation is about five times higher for sites ipnA3 or ipnA4AB and 50 times higher for site ipnA1 than that required for ipnA2. We conclude that the relative affinity of the sites in the order ipnA2:[ipnA3, ipnA4AB]:ipnA1 is 50:10:1.

Deletion Analysis of the Highest Affinity Site, ipnA2—ipnA transcription is subjected to carbon and pH regulation through independent mechanisms, and either a derepressing carbon source or ambient alkaline pH is sufficient for elevated *ipnA* transcription (9, 13). Therefore, alkaline ambient pH or alkalinity-mimicking *pacC*^{c} mutations override the effects of a repressing carbon source and result in elevated *ipnA* message levels on sucrose (9). Because *pacC*^{c} mutations represent the gain-of-function class, if PacC binding sites at the *ipnA* promoter were physiologically functional, their targeted mutational destruction should result in impaired promoter activity. We also reasoned that deletion of the highest affinity site, ipnA2, should have the strongest effect. Therefore, we precisely deleted by PCR a 21-bp fragment (−601 to −579 from the major transcription start point) that contained the ipnA2 PacC binding site. A fragment carrying this mutation was used to replace the corresponding wild-type fragment in plasmid pBSΔ1, in which the *ipnA* promoter drives expression of a translational fusion with *lacZ* (10), denoted *ipnA*^{p}::*lacZ*. The resulting plasmid was transformed into *A. nidulans*, and transformants carrying single-copy integration events at either the *ipnA* or *argB* locus were identified using Southern analysis. These transformants were purified and grown with sucrose as the main carbon source under either alkaline or acidic conditions (see "Experimental Procedures"), and expression of the wild-type (strains [*ipnA*^{p}::*lacZ*]::*argB* and [*ipnA*^{p}::*lacZ*]::*ipnA*) or mutated (strains [*ipnA*^{p}(-2)::*lacZ*]::*argB* and [*ipnA*^{p}(-2)::*lacZ*]::*ipnA*) *lacZ* fusion genes integrated in either chromosomal locus was monitored by assaying β-galactosidase in mycelial extracts (Fig. 3). Promoter activity was substantially higher under alkaline than under acidic conditions (as expected from the substantial elevation in *ipnA* mRNA levels found in mycelia grown under alkaline conditions despite the presence of a strongly repressing carbon source (9)). However, under alkaline conditions (*i.e.* under conditions in which the PacC transcription factor is functional), the mutant *ipnA* promoter deleted for the ipnA2 PacC binding site showed roughly half the activity of the wild-type promoter (the wild-type and mutant *ipnA*::*lacZ* fusion genes resident at *argB* reproducibly showed 50% of the activity of those integrated at *ipnA*, possibly due to position effects on gene expression). By contrast, under acidic conditions (in which PacC shows reduced function), the mutant promoter did not show the above 50% reduction in activity (readers should note that expression of the *ipnA*::*lacZ* gene at *argB* is largely prevented by acidic growth conditions). These results indicate that although the *ipnA* promoter is at least partially under direct PacC control, the contribution of the highest affinity site, ipnA2, to transcription elevation under ambient alkaline pH is relatively modest, at least in the presence of the lower affinity sites ipnA3 and ipnA4AB.

Effect of the ipnA2 Deletion in a pacC^c *Genetic Background*—The effects on *pacC* function of alkaline growth conditions achieved by buffering the culture media are never so extreme as those caused by alkalinity-mimicking *pacC*^{c} mutations at any ambient pH condition (5, 6). This could have resulted in underestimation in the above experiments of the contribution of the highest affinity site, ipnA2, to *ipnA* transcription, and we reasoned that perhaps more extreme alkaline conditions might reveal a more crucial role of this site. Therefore, we used parasexual genetics to construct strains carrying either a wild-type or a mutant *ipnA*^{p}(-2)::*lacZ* fusion gene at *argB* in a *pacC*^{c}14 background (parasexual genetics rather than the meiotic cycle were used in these crosses to prevent loss of the reporter fusion by a meiotic recombination event involving duplicate copies of *argB*). Fig. 4 shows that expression of the wild-type fusion under acidic conditions is 12–14-fold higher in a *pacC*^{c}14 background (two independent strains were tested) than in a *pacC*$^+$ background. Again, the mutated fusion showed half the activity of the wild type (two independent clones were also tested). This confirmed that site ipnA2 contributes to 50% of the *ipnA*^{p} activity under alkaline conditions. In passing, this experiment shows that changes in β-galactosidase activity resulting from alkaline growth conditions are mediated by *pacC* and do not reflect indirect physiological effects resulting from differences in growth pH. In addition, this result formally confirms that the active PacC version is a transcriptional activator of *ipnA*, as proposed by Espeso *et al.* (9), Tilburn *et al.* (5), and Orejas *et al.* (6).

Site-directed Mutagenesis of Sites ipnA3 and ipnA4AB—To test the contribution of the two other binding sites to promoter activation by PacC, an $A_4 \rightarrow T$ site-destroying mutation, which abolishes PacC binding to its cognate sequence (5), was introduced in either ipnA3 or ipnA4AB. (In fact, two such mutations were made in this latter double site, one in each of the single hexanucleotides.) The corresponding promoter fragments with these point mutations were fused to *lacZ*, and the constructs were integrated in single copy at *argB* (strains [*ipnA*^{p}(-3)::*lacZ*]::*argB* and [*ipnA*^{p}(-4AB)::*lacZ*]::*argB*, respectively). Promoter activity was tested under both acidic and alkaline conditions (Fig. 5). These mutations caused no effects under acidic conditions (under which wild-type (see above) and mutant promoter activity was very low, as measured using the *ipnA*::*lacZ* fusion genes integrated at *argB*). By contrast, under alkaline conditions, mutation of either site reduced promoter activity. Loss of PacC binding to ipnA4B caused a similar effect to that of ipnA2 deletion (*i.e.* a reduction to 50% of the wild-type promoter activity), whereas inactivation of ipnA3 reduced activity to 20% of the wild type. We conclude that the three sites (ipnA2, ipnA3, and ipnA4AB) are physiologically functional PacC binding sites, but none of these sites is by itself essential

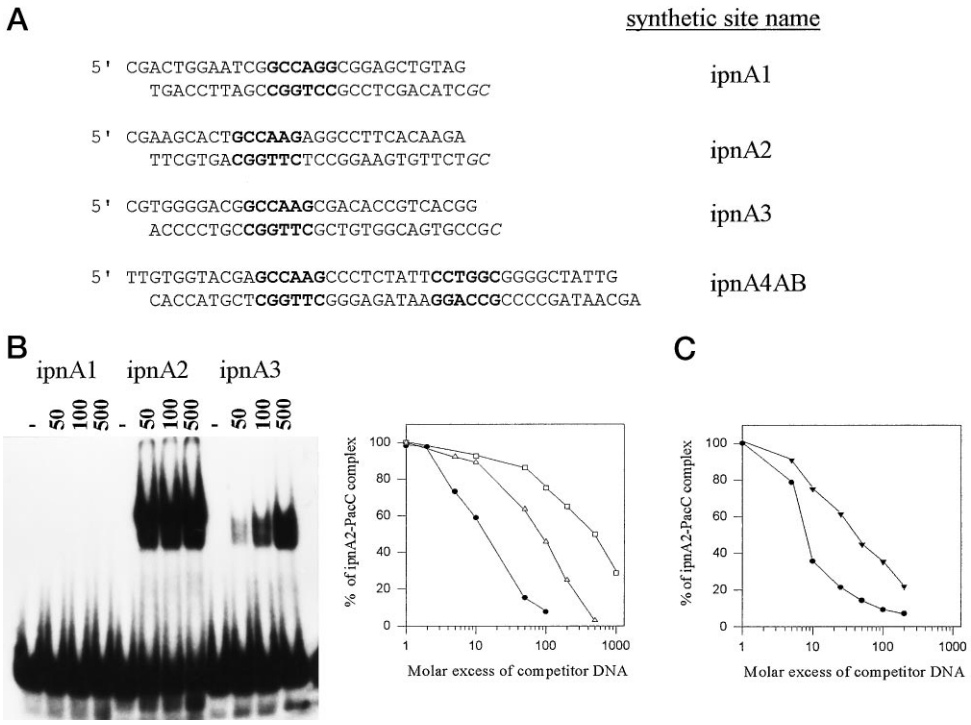

FIG. 2. **Relative affinities *in vitro* of PacC binding sites.** *A*, synthetic double-stranded oligonucleotides used in this work, corresponding to the indicated sites. The core PacC binding hexanucleotide is shown in *bold*. Nucleotides in *italics* (*right ends* of synthetic sites ipnA1, ipnA2, and ipnA3) do not correspond to the *ipnA*^{p} sequence and were introduced to facilitate subcloning and end-labeling of these sites. *B*, mobility shift assays using the indicated ^{32}P-labeled synthetic PacC binding sites (~15 fmol/binding assay) and different amounts (in nanograms) of the purified GST::PacC(30–195*) fusion protein. 1 ng of fusion protein is approximately equal to 22 fmol. *C*, competition of the formation of a protein-DNA complex between the GST::PacC fusion protein and the ^{32}P-labeled ipnA2 binding site by an excess of each of the other unlabeled PacC binding sites. The amount of label present as protein-DNA complex in each lane was quantified using a PhosphorImager (Molecular Dynamics) and given as a percentage of that measured in a control reaction carried out in the absence of competitor DNA. Each reaction contained approximately 220 fmol of purified fusion protein and 15 fmol of labeled ipnA2 site. Symbols are as follows: ●, competition with cold ipnA2; △, competition with ipnA3; ▲, competition with ipnA4AB; □, competition with ipnA1.

for promoter activation by PacC under alkaline conditions.

The Effects of Double and Triple Mutations—To analyze the individual contribution of each site to promoter activity and confirm that sites ipnA2, ipnA3, and ipnA4 are sufficient to account for the activation of *ipnA*^{p} under alkaline conditions, we introduced all possible combinations of double mutations and a triple mutation in the promoter of the *ipnA::lacZ* gene and selected transformants with each of the corresponding constructs integrated in single copy at *argB*. All of these mutated promoters showed basal levels of activity under acidic conditions. Under alkaline conditions, simultaneous inactivation of ipnA2 and ipnA3 (strain [*ipnA*^{p}(-2, -3)::*lacZ*]::*argB*) showed the same effect as that of ipnA3 alone, *i.e.* a 5-fold reduction in promoter activity (Fig. 5). A similar effect was obtained by simultaneous inactivation of ipnA2 and ipnA4 (strain [*ipnA*^{p}(-2, -4)::*lacZ*]::*argB*), indicating that ipnA3 and ipnA4 have a similar contribution to promoter activity in the absence of the other binding sites. By contrast, simultaneous destruction of ipnA3 and ipnA4 (strain [*ipnA*^{p}(-3, -4)::*lacZ*]::*argB*) reduced promoter activity to 10% of the wild type, in agreement with the less important contribution deduced above for site ipnA2. Inactivation of all three sites (strain [*ipnA*^{p}(-2, -3, -4)::*lacZ*]::*argB*) abolished the elevation in promoter activity resulting from alkaline growth conditions.

DISCUSSION

We had previously described the presence of four PacC binding sites in the promoter of the penicillin biosynthesis structural gene *ipnA*. We show here that three of these binding sites are physiologically functional and mediate direct activation of *ipnA* transcription by PacC under alkaline conditions, in agreement with elevated levels of *ipnA* mRNA detected in mycelia from alkaline growth conditions or carrying alkalinity-mimicking *pacC*^{c} mutations. This substantiates our previous proposal that PacC is a transcriptional activator for alkaline-expressed genes (5, 6, 9).

In the current model (5), the active PacC version represses transcription of acid-expressed genes. The activating and repressing mechanisms of PacC might differ substantially (16). The promoter of the acid-expressed gene *pacA*, encoding an acid phosphatase, has no 5′-GCCARG-3′ sites in the 1.3-kilobase pair region upstream of the initiation codon. Genetic analysis has identified a gene, denoted *pacM*, involved in the repression of acid phosphatase but not in the activation of alkaline phosphatase synthesis (16). *pacM* could therefore encode a repressor or a protein interacting with PacC for the repression of acid-expressed genes (16).

The integrity of all three physiological binding sites is essential for full transcriptional activation by PacC. Triple inactivation of the sites nearly abolished promoter activity, demonstrating that these three PacC binding sites are sufficient for pH-dependent activation. In fact, the triple mutant promoter showed, under alkaline conditions, lower activity than the wild type under acidic conditions, indicating that even under the latter conditions (which are not extremely acidic) some PacC-mediated activation takes place. This is in agreement with the presence of detectable levels of *pacC* mRNA (5) and substantial levels of the active PacC form in acid grown mycelia (6) because the acidic conditions used are less extreme than those resulting, for example, from mutational inactivation of the *pal* signal transduction pathway. Single inactivation of the sites revealed that, somewhat unexpectedly, the highest affinity site, ipnA2, has a modest role in transcriptional activation, contributing to

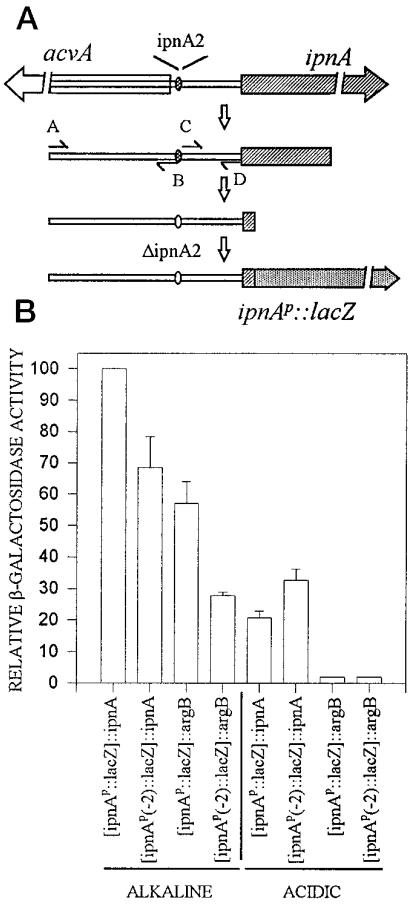

FIG. 3. **Inactivation of the ipnA2 highest affinity PacC binding site.** *A*, construction of a mutated *ipnA::lacZ* gene in which a 21-bp sequence that includes the DNase I footprint corresponding to site ipnA2 was deleted. Separate PCR amplification reactions using primer pairs A-B and C-D (described in detail under "Experimental Procedures") were used to obtain two fragments that, when ligated in the correct orientation, reconstructed an *ipnA*^{P} precisely deleted for this sequence. *B*, β-galactosidase activity in strains carrying a wild-type or a mutated copy of the *ipnA*^{P}::*lacZ* fusion integrated at either *ipnA* or *argB* (see "Experimental Procedures") and grown under either acidic or alkaline conditions, as indicated. Activities are expressed as a percentage of that shown by a strain carrying an *ipnA*^{P}::*lacZ* fusion gene integrated at *ipnA* grown under alkaline conditions. The values represent the average of four independent experiments.

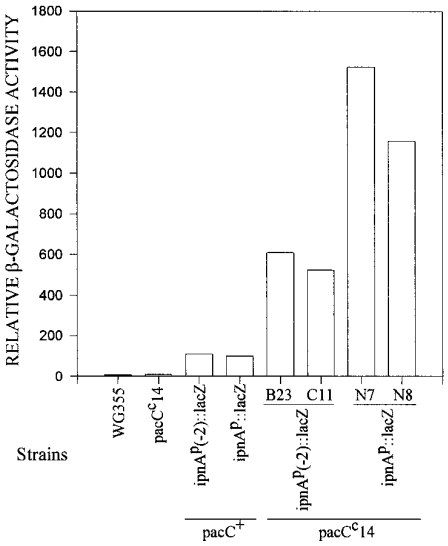

FIG. 4. **Effects of an alkalinity-mimicking *pacC*^{c} mutation on expression of wild-type and mutant (deleted for ipnA2) *ipnA*^{P}*::lacZ* fusion genes integrated at *argB*.** β-Galactosidase activities in mycelial extracts of the indicated strains are shown relative to that found in a *pacC*$^{+}$ strain carrying the wild-type *ipnA*^{P}::*lacZ* fusion gene. Results for two independently isolated *pacC*^{c} progeny carrying either the wild-type *ipnA*^{P}::*lacZ* (strains *N7* and *N8*) or the mutant *ipnA*^{P}(-2)::*lacZ* (strains *B23* and *C11*) fusion genes are shown. *WG355*, the recipient strain used for transformation of *ipnA*^{P}::*lacZ* constructs. *pacC*^{c}*14*, the relative activity in extracts from the parental strain carrying this allele used to make the diploid. All strains were grown under acidic conditions.

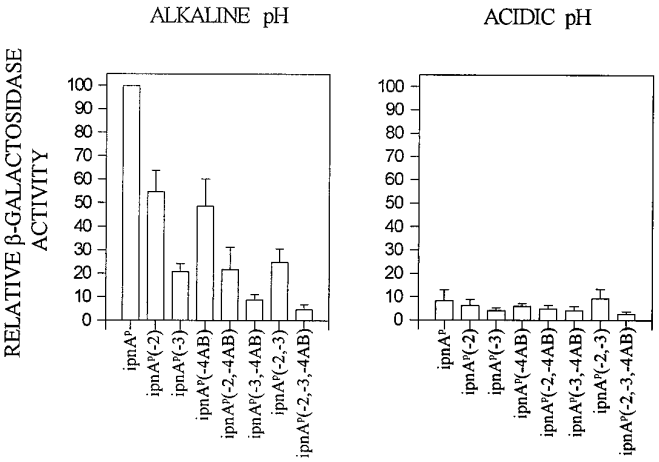

FIG. 5. **Effects of single, double, and triple inactivating mutations in PacC binding sites of the *ipnA*^{p} under alkaline or acidic growth conditions.** β-Galactosidase activities in mycelial extracts of the indicated strains and growth conditions were measured and given as a percentage of that corresponding to a wild-type *ipnA*^{P}::*lacZ* fusion gene under alkaline growth conditions. All constructs were integrated in single copy at *argB*. For simplicity, only the relevant features of each promoter (in all cases fused in an identical way to *lacZ*) are shown *below* the corresponding bar. *Bars*, the average values obtained from three separate cultures for each strain.

only 50% of promoter activity in the presence of the other PacC binding sites. The presence of ipnA2 alone results in a roughly 2-fold elevation of basal promoter activity in response to alkaline pH.

By contrast, single inactivation of ipnA3 (which has a 5-fold lower affinity than ipnA2) caused a significant decrease in promoter activity, and a mutant promoter lacking this site shows a 5-fold decrease in activity under alkaline pH conditions. The double ipnA4AB site was less important in the presence of ipnA3 but almost essential in its absence. The presence of either ipnA3 or ipnA4AB alone accounts for a 4–5-fold elevation over basal promoter activity. We conclude that sites ipnA3 and ipnA4, showing a 5-fold lower affinity *in vitro* for PacC than ipnA2, are physiologically more important for *ipnA* transcription than the latter, perhaps suggesting that specific contacts between PacC and the general transcription factors at the *ipnA* transcription start site are more favored when PacC is bound to the former sites than to the latter. In this context, we note that ipnA2 is the most distal site from the *ipnA* gene. Therefore, it is possible that the ipnA2 site might have a more relevant role in expression of the divergently transcribed *acvA* gene, which is also pH-regulated.[2] It should also be noted that the affinity of the GST::PacC(30–195*) fusion protein used here for its cognate sites is not significantly different from that shown by PacC from *A. nidulans* extracts (6), ruling out the possibility that the bacterially expressed fusion protein does not accurately reflect the PacC binding specificity.

We have no indication of cooperativity between PacC binding

[2] E. A. Espeso and M. A. Peñalva, unpublished results.

sites at the *ipnA* promoter. Xu *et al*. (17) have shown that a single GAL4 dimer is able to maximally activate transcription of a target gene. The presence of several PacC binding sites at the *ipnA* promoter would simply increase the chance of having at least one productive binding event.

The *ipnA* promoter is under pH and carbon-source regulation. Favored carbon sources repress *ipnA* transcription (10, 13, 18). However, as utilization of repressing carbon sources results in media acidification, it was formally possible that carbon repression would result from the absence of PacC activation at an acidic ambient pH. We show here (as proposed by Espeso *et al*. (9)) that physiological positive-acting PacC binding sites are not included in the region shown by Pérez-Esteban *et al*. (10) to be involved in carbon regulation through a negative-acting mechanism. These data strongly support our conclusion (9) that pH and carbon regulation are mechanistically independent. Moreover, in the related fungus *P. chrysogenum*, *pcbC* (the isofunctional homologue of *ipnA*) gene transcription also seems to be regulated by carbon repression and PacC. However, in contrast to the situation in *A. nidulans*, ambient alkaline pH does not override the negative effects on *pcbC* transcription of utilization of a repressing carbon source (8). In full agreement with the proposed dual mechanism, the negative effects on *ipnA* transcript levels of a repressing carbon source are additive with those caused by loss-of-function mutations *palA*1, *palB*7, and *palC*4 preventing the formation of the functional PacC form (2, 3, 5, 6) or with those caused by $pacC^{+/-}$508 (5), a loss-of-function mutation in PacC (data not shown).

Acknowledgments—We thank Herbert N. Arst, Jr. and Joan Tilburn for continuous discussions, encouragement, and critical reading of the manuscript; Beatriz Pérez-Esteban for advice on β-galactosidase assays; and Elena Reoyo for technical assistance.

REFERENCES

1. Rossi, A., and Arst, H. N., Jr. (1990) *FEMS Microbiol. Lett.* **66,** 51–54
2. Caddick, M. X., Brownlee, A. G., and Arst, H. N., Jr. (1986) *Mol. & Gen. Genet.* **203,** 346–353
3. Arst, H. N., Jr., Bignell, E., and Tilburn, J. (1994) *Mol. & Gen. Genet.* **245,** 787–790
4. Denison, S. H., Orejas, M., and Arst, H. N., Jr. (1995) *J. Biol. Chem.* **270,** 28519–28522
5. Tilburn, J., Sarkar, S., Widdick, D. A., Espeso, E. A., Orejas, M., Mungroo, J., Peñalva, M. A., and Arst, H. N., Jr. (1995) *EMBO J.* **14,** 779–790
6. Orejas, M., Espeso, E. A., Tilburn, J., Sarkar, S., Arst, H. N., and Peñalva, M. A. (1995) *Genes & Dev.* **9,** 1622–1632
7. MacCabe, A. P., Vandenhombergh, J. P. T. W., Tilburn, J., Arst, H. N., Jr., and Visser, J. (1996) *Mol. & Gen. Genet.* **250,** 367–374
8. Suárez, T., and Peñalva, M. A. (1996) *Mol. Microbiol.* **20,** 529–540
9. Espeso, E. A., Tilburn, J., Arst, H. N. J., and Peñalva, M. A. (1993) *EMBO J.* **12,** 3947–3956
10. Pérez-Esteban, B., Orejas, M., Gómez-Pardo, E., and Peñalva, M. A. (1993) *Mol. Microbiol.* **9,** 881–895
11. Clutterbuck, A. J. (1993) in *Genetic Maps, Locus Maps of Complex Genomes* (O'Brien, S. J., ed) 6th Ed., Vol. 3, pp. 3.71–3.84, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
12. Cove, D. J. (1966) *Biochim. Biophys. Acta* **113,** 51–56
13. Espeso, E. A., and Peñalva, M. A. (1992) *Mol. Microbiol.* **6,** 1457–1465
14. Tilburn, J., Scazzocchio, C., Taylor, G. G., Zabicky-Zissman, J. H., Lockington, R. A., and Davies, R. W. (1983) *Gene (Amst.)* **26,** 205–211
15. Espeso, E. A., and Peñalva, M. A. (1994) *FEBS Lett.* **342,** 43–48
16. Sarkar, S., Caddick, M. X., Bignell, E., Tilburn, J., and Arst, H. N., Jr (1996) *Biochem. Soc. Trans.* **24,** 360–363
17. Xu, H. E., Kodadek, T., and Johnston, S. A. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 7677–7680
18. Brakhage, A. A., Browne, P., and Turner, G. (1992) *J. Bacteriol.* **174,** 3789–3799
19. MacCabe, A. P., van Liempt, H., Palissa, H., Unkles, S. E., Riach, M. B. R., Pfeifer, E., von Döhren, H., and Kinghorn, J. R. (1991) *J. Biol. Chem.* **266,** 12646–12654

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

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J. Biol. Chem. 1996, 271:28825-28830.
doi: 10.1074/jbc.271.46.28825