Pgas, a Low-pH-Induced Promoter, as a Tool for Dynamic Control of Gene Expression for Metabolic Engineering of *Aspergillus niger*

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ABSTRACT The dynamic control of gene expression is important for adjusting fluxes in order to obtain desired products and achieve appropriate cell growth, particularly when the synthesis of a desired product drains metabolites required for cell growth. For dynamic gene expression, a promoter responsive to a particular environmental stressor is vital. Here, we report a low-pH-inducible promoter, *Pgas*, which promotes minimal gene expression at pH values above 5.0 but functions efficiently at low pHs, such as pH 2.0. First, we performed a transcriptional analysis of *Aspergillus niger*, an excellent platform for the production of organic acids, and we found that the promoter *Pgas* may act efficiently at low pH. Then, a gene for synthetic green fluorescent protein (*sgFP*) was successfully expressed by *Pgas* at pH 2.0, verifying the results of the transcriptional analysis. Next, *Pgas* was used to express the *cis*-aconitate decarboxylase (*cad*) gene of *Aspergillus terreus* in *A. niger*, allowing the production of itaconic acid at a titer of 4.92 g/liter. Finally, we found that *Pgas* strength was independent of acid type and acid ion concentration, showing dependence on pH only.

IMPORTANCE The promoter *Pgas* can be used for the dynamic control of gene expression in *A. niger* for metabolic engineering to produce organic acids. This promoter may also be a candidate tool for genetic engineering.

KEYWORDS *Aspergillus niger*, low-pH-inducible promoter, itaconic acid, dynamic gene expression, metabolic engineering

Aspergillus niger is an excellent cell factory for the production of organic acids (1). The rational engineering of *A. niger* has attracted increasing attention, and great achievements have been made in this area (2, 3). Nevertheless, because heterologous pathways are controlled by constitutive promoters, e.g., the promoter of the glyceraldehyde-3-phosphate dehydrogenase gene (*gpdA*), most of this research has focused on static metabolic engineering; that is, the gene expression level is set without sensing changes in the pathway output or cellular environment (4). One disadvantage of static control is related to the trade-off between growth and the production of a desired compound; suboptimal productivity is obtained because these pathways can drain metabolites required for biomass synthesis (5). Metabolic engineering approaches are being developed to tackle this problem via a dynamic control system to compensate for changing conditions (6). In this system, the cell modulates its metabolic pathways dynamically to adjust fluxes such that the required metabolic intermediates are delivered at the appropriate levels and times to optimize growth (7).

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FIG 1 RNA expression levels of 7 genes that were candidates to be acid-inducible genes. (A) Relative fold change of the most upregulated genes from pH 4.5 to pH 2.5 for the protein [GSE11725](#). (B) FPKM change of 6 of the 7 genes during citrate fermentation for the protein [GSE74544](#).

Although there are many inducible promoters, e.g., the glucoamylase promoter *Pgla* (8, 9), xylanase promoter *Pxln* (10), Taka-amylase A promoter *Pamy* (11), alcohol dehydrogenase promoter *Palc* (12), and the tetracycline (Tc)-regulated gene expression system (Tet-on system) (13), these systems have not been successfully applied to the dynamic control of gene expression for the metabolic engineering of *A. niger*. It is necessary to add a specific substrate (e.g., xylose or doxycycline) to induce gene expression, making these promoters useful for scientific purposes (14) but not feasible for industrial purposes. Furthermore, *Pxln* is inhibited by glucose, and the strength of *Pgla* decreases as glucose is exhausted, resulting in unstable expression in medium with glucose as the main carbohydrate (15). During organic acid production by *A. niger*, e.g., in citrate fermentation, the pH of the medium decreases from 5.0 to below 2.0 and is maintained below 2.0 in later phases. Therefore, it would be advantageous to identify a low-pH-inducible promoter for dynamic metabolic engineering of *A. niger*. Here, we report a low-pH-inducible promoter, *Pgas*, which promoted gene expression efficiently at low pHs, such as pH 2.0. The induction pattern of the promoter *Pgas* was characterized, and this promoter was used to engineer *A. niger* for the production of itaconic acid. As a promoter induced completely in response to low pH in fungi, this promoter may have potential applications in the production of organic acids by *A. niger* and other microbes.

RESULTS

Selection of low-pH-induced promoters. We first analyzed *A. niger* gene expression data (accession number [GSE11725](#)) (16) from *A. niger* BO-1, which was obtained from Novozymes A/S (Kalundborg, Denmark) to detect changes in mRNA levels from pH 4.5 to pH 2.5. The upregulated genes with the greatest fold changes were *fnx*, *dhy*, *gas*, *patl*, *amy*, *pth*, and *aat* (Fig. 1A; see also Table 4, below), among which the gene *dhy* was absent from the genome of H915-1 (accession number [LLBX00000000](#)). Transcriptome analysis ([GSE74544](#)) of the industrial strain *A. niger* H915-1, which is used for citrate

FIG 2 sGFP expression induced by *Pgas*. (A) sGFP expression under the *gas* and *gpdA* promoters in *A. niger* hyphae at different pHs. Bar, 20 μ m. WT, wild-type. (B) Relative sGFP fluorescence intensity of transformants at different pH levels.

production, at 6, 12, 24, 36, and 48 h revealed that *fnx*, *gas*, and *amy* were upregulated during citrate secretion and that *gas* was upregulated with the highest number of fragments per kilobase of transcript per million mapped reads (FPKM) (Fig. 1B). The *gas* gene encodes the 1,3-beta-glucanotransferase GelD, which plays a crucial role in fungal cell wall maintenance by synthesizing the main wall component, glucan (17). Gene expression may need to be enhanced at low pH, which may destroy glucan (18). Therefore, we selected the *gas* promoter as a candidate low-pH-inducible promoter. The sequence 1,500 bp upstream of the start codon ATG was analyzed using the Neural Network Promoter Prediction software (version 2.2), and the region from bp 153 to 203 was predicted as a transcription start site with a score of 0.98.

***Pgas*-induced sGFP expression at pH 2.0.** The expression strength of *Pgas* was compared with that of *PgpdA*, a strong constitutive promoter used for the engineering of *A. niger*. For synthetic green fluorescent protein (sGFP) expression, a human codon-optimized S65T mutant was used. The human codon-optimized sGFP has been shown to be well expressed in many higher eukaryotic systems, including fungi and plants (19, 20). The transformants used were all single-copy strains identified through quantitative PCR (qPCR). The conidia of *Pgas*-sGFP and *PgpdA*-sGFP were treated in LBL medium within a pH gradient, and the mycelia were analyzed under blue light. Wild-type H915-1 did not show fluorescence at pH 2.0 or pH 5.0. In contrast, *PgpdA*-sGFP showed a high fluorescence intensity at pH 5.0 but much less fluorescence at pH 2.0. The *Pgas*-sGFP transformant showed minimal sGFP expression at pH 5.0 but enhanced fluorescence intensity at pH 2.0, indicating that the promoter acted as an acid-enhanced promoter (Fig. 2A). Because random integration via nonhomologous end joining (NHEJ) was used for gene integration, the integration locus may have had an impact on the gene expression level. Accordingly, we selected 10 transformants and checked their fluorescence intensities. The results indicated that these transformants had almost the same fluorescence intensity (data not shown).

The fluorescence intensities of *Pgas*-sGFP and *PgpdA*-sGFP were further analyzed using a Cytation 3 cell-imaging multimode reader (BioTek, Winooski, VT, USA). The fluorescence intensity of *PgpdA*-sGFP at pH 5.0 was set as 100%. As shown in Fig. 2B, the fluorescence of *PgpdA*-sGFP decreased from pH 5.0 to pH 2.0, and the fluorescence intensity was maintained at 60% at pH 2.0. Nevertheless, *Pgas*-sGFP showed little fluorescence at pH 3.0, 4.0, and 5.0 but enhanced fluorescence at pH 2.0, which was 15% stronger than that of *PgpdA*-sGFP at pH 2.0. *Pgas* did not promote gene expression at pH 5.0 but promoted gene expression to the same level as *PgpdA* at pH 2.0; accordingly, this promoter was deemed more suitable for the dynamic engineering of *A. niger* for organic acid production.

The CAD expression induced by *Pgas* conferred *A. niger* H915-1 with the ability to produce itaconate. We used *Pgas* to engineer *A. niger* to produce the C₅ dicarboxylic acid itaconate, one of 12 building block chemicals selected by the U.S. Department of Energy (21). *A. niger* is a natural citrate producer that cannot synthesize itaconate because it lacks CAD in the pathway (Fig. 3A) (22). In this study, the CAD gene was fused with a mitochondrion-directed signal at the N terminus, because mitochondrial localization significantly improves itaconate production (23). After verification of gene copy number, transformants with a single copy of the gene were used to perform itaconate fermentation. Itaconate fermentation was carried out with synthetic medium (24) at an initial pH of 3.1 in shake flasks for 6 days. All transformants still produced citrate as the main product, but as the gene integrations through NHEJ may cause a different gene insertion locus, leading to different gene expression levels of neighbor genes, the citrate titers of different strains showed large differences. *PgpdA*-sCAD produced itaconate at around 0.76 and 0.82 g/liter. Nevertheless, CAD expression induced by *Pgas* conferred *Pgas*-sCAD transformants with the ability to produce itaconate at titers of 4.34 and 4.92 g/liter, respectively (Fig. 3B), which was around 5-fold higher than that produced by *PgpdA*-sCAD. Further analysis of the fermentation profile showed a gradual increase in sCAD expression in *Pgas*-sCAD-2 that was accompanied by a decrease in broth pH (Fig. 3C). The pH of the medium, which was adjusted to 3.1 for fermentation, dropped to 2.46 at 8 h after inoculation and further decreased to 1.72, 1.57, 1.38, and 1.37 at 24, 48, 84, and 108 h, respectively. sCAD expression in the presence of *Pgas* increased to 2.37-, 2.91-, 2.99-, and 3.23-fold at 24, 48, 84, and 108 h, respectively, above that at 8 h. In contrast, the expression of sCAD in the presence of the *PgpdA* promoter decreased to around 20% during itaconate fermentation compared with that at 8 h. The enhanced sCAD expression level in *Pgas*-sCAD-2 resulted in a 5.3-fold-higher itaconate titer than that of *PgpdA*-sCAD-1 at 108 h. The use of *Pgas* enabled dynamic control of gene expression during the production phase, resulting in efficient organic acid production.

Characterization of the *Pgas* induction pattern. We analyzed the CAD expression pattern under *Pgas* by using *Pgas*-sCAD-2; in particular, we examined the influence of five acid types (acetic acid, citric acid, phosphoric acid, hydrochloric acid, and sulfuric acid) on the induction capacity. As shown in Fig. 4A, greater induction was observed for organic acids than for inorganic acids. CAD expression induced by acetic acid was 1.3 times higher than that induced by phosphoric acid, and the induction strength of citrate was 19% higher than that of phosphoric acid. The least effective inducer was hydrochloric acid, whose induction strength was 77% that of phosphoric acid. Nevertheless, the relative changes for all five acids were less than 2-fold, indicating that the influence of acid type on *Pgas* induction is limited.

All solutions were adjusted to pH 2.0; accordingly, the concentrations of the five acids were different. It was therefore necessary to examine the influence of acid concentration on CAD expression. When the pH was not adjusted, the solution pH values for 20, 40, and 80 g/liter citrate were 2.71, 2.41, and 2.10, respectively, and the relative expression level of CAD increased as the pH decreased. Nevertheless, for citrate buffer at concentrations of 20, 40, and 80 g/liter and with adjustment to pH 3.0, the

FIG 3 Shake flask culture for itaconate fermentation of CAD transformants. (A) Schematic for citrate and itaconate production of *A. niger*. The dotted line (with arrowhead) indicates an exogenous pathway that does not exist in the natural strain. Abbreviations: GLU, glucose; PYR, pyruvate; CA, citrate; ACO, *cis*-aconitate; IA, itaconate; ICA, isocitrate; CAD, *cis*-aconitate decarboxylase. (B) Itaconate titers of different CAD transformants after 6 days of cultivation. Error bars represent the standard errors of the means of three biological replicates. (C) Itaconate fermentation of Pgas-sCAD-2 (black symbols) and Pgpda-sCAD-1 (white symbols). Circles indicate broth pH; stars indicate relative fold changes of sCAD expression compared to the expression level of Pgpda-sCAD-1 at 8 h; squares represent itaconate titers; triangles represent citrate titers.

relative CAD expression levels were almost the same, indicating that the acid concentration had no significant impact on the capacity to induce *Pgas* (Fig. 4B).

The mRNA levels of CAD under different pH conditions were further analyzed. The expression level at pH 7.0 was set as 100%. As the pH decreased, the CAD expression level increased (Fig. 4C). Transcription levels at pH 6.0, 5.0, and 4.0 increased slowly, but the expression level at pH 3.0 increased 15-fold above that at pH 7.0. In addition, the expression level at pH 2.0 increased sharply by 122-fold, indicating that *Pgas* was a low-pH-induced promoter and that a new transduction pathway may exist (25). The relationship between the relative expression level and pH was calculated by linear regression analysis using the following equation: $\log(E) = [(6.042/\text{pH}) - 0.9059]$, where E is the relative expression level compared with expression at pH 7.0; the correlation coefficient factor (R^2) is 0.9962.

FIG 4 Factors affecting relative *Pgas*-inducing capacity. (A) Influence of acid type on *Pgas*-inducing capacity. (B) Influence of citrate concentration on *Pgas*-inducing capacity. (C) Fold changes in CAD expression at different pHs. Error bars represent the standard errors of the means of results for three biological replicates.

Identification of a *Pgas* regulator via DNA pulldown assays. *Pgas* was the first promoter found to be induced by extremely low pH in eukaryotes, and the regulatory mechanism was investigated in DNA pulldown assays (Fig. 5). For samples at pH 7.0, the pulldown protein bands were the same as those of the control. However, for samples at pH 2.0, compared with proteins showing nonspecific binding to the control probe the proteins binding to the *Pgas* probe were separated with two additional bands with molecular masses around 60 and 70 kDa; these bands were also not present in samples at pH 7.0, indicating the great regulatory potential of *Pgas*. The two proteins were identified through liquid chromatography-tandem mass spectrometry (LC-MS/MS) to have masses of 72 kDa (accession no. [XP_001388781.2](#)) and 60 kDa (accession no. [XP_001396281](#)), and both were predicted to be transcriptional regulators with unknown function.

DISCUSSION

A. niger is a potential host strain for biorefinery applications. Owing to its saprophytic lifestyle, it is able to utilize a broad substrate range of nutrients, from monosaccharides to polymers, such as cellulose (26). In addition, it naturally produces acids

FIG 5 DNA pulldown assay for identification of a transcription regulator on *Pgas*. (A) Flow chart presenting the DNA pulldown assay process. (B) DNA pulldown assay results. Lanes: M, protein marker; 1, sample at pH 2.0 in step 5; 2, sample at pH 7.0 in step 5; 3, sample at pH 2.0 in step 3 before addition to Dynabeads; 4, sample at pH 7.0 in step 3 before addition to Dynabeads; 5, sample at pH 7.0 in step 10; 6, sample at pH 2.0 in step 10. Two white arrows point to protein bands expressed in the nucleus only at pH 2.0.

and enzymes and can synthesize a variety of secondary metabolites (27), some of which can be applied as pharmaceuticals. Furthermore, this organism has a long history of safe use and can be applied for large-scale fermentation technology (28). Finally, there are many available biology-based tools for metabolic engineering, which involves either static control or inducible control according to artificially designated gene expression changes. Compared with static control or inducible control, dynamic control can regulate gene expression naturally through the host strain to achieve the proper metabolite flux; this may lead to better fermentation performance (4). During organic acid fermentation by *A. niger*, e.g., citrate fermentation, several prerequisite conditions

need to be met, including an abundant carbon source, excessive dissolved oxygen, and low pH (1). Citrate fermentation can be divided naturally into two parts according to the pH. During the first period, spore germination requires a pH of more than 5, and hyphal growth requires an optimal pH of 3.8 to 6.0. Diverse organic acids are secreted during hyphal grow; as a result, the pH of the medium drops dramatically to around 2.0 within a few hours of the initiation of fermentation. During the second period, the pH for citrate production needs to be low (pH, <2). The pH can be a natural switch for gene expression if a low-pH-inducible promoter is found.

Low-pH-inducible promoters, which can be induced at pH 5.5 but not at pH 7.0, have already been studied in *Lactococcus lactis*, a lactic acid bacterium tolerant of the acidic environments encountered in either fermented foods or the gastrointestinal tract (29). In addition, a *cis*-acting ACID box and transcriptional regulator were identified (30). Nevertheless, reported differences in the nucleotide compositions of promoter sequences (31) and the mechanisms of translation initiation (32) between prokaryotes and eukaryotes have highlighted the importance of studies promoters in *A. niger* that are induced by extremely low pH. In this study, the extremely low-pH-induced promoter *Pgas* was identified. The promoter induced a 1,3-beta-glucanosyltransferase to facilitate the synthesis of glucan, the main component of the fungal cell wall, and played an important role in the stress response to strong acids. Furthermore, the *Pgas* expression pattern was analyzed through analysis of both *sGFP* and *sCAD* expression. *Pgas* was found to be obviously expressed only at pH 2.0, with a strength similar to that of *PgpdA*. In addition, the *Pgas* strength was influenced somewhat by the type and concentration of acid, but it was only induced according to pH. The existence of a relationship between relative expression and pH made this promoter an ideal candidate for fine-tuning. Additionally, the precise control of gene expression may be useful for uncovering the relationship between fungal genomes and phenotypes in the academic setting.

Because *Pgas* was verified as a low-pH-induced promoter, the *sCAD* transformant was used for itaconate fermentation. *Aspergillus terreus* was the natural producer of itaconate, with a production level of 80 g/liter. After the gene encoding the key enzyme AtCAD was identified in 2008 (22), many studies were performed to utilize this gene to produce itaconate in various hosts, including *Escherichia coli* (33), *Corynebacterium glutamicum* (34), *Saccharomyces cerevisiae* (35), *Yarrowia lipolytica* (36), and *A. niger*. Because *A. niger* produced large amounts of citrate (180 g/liter), the precursor of itaconate, there was great interest in transforming the natural citrate producer into an itaconate producer; however, constitutive expression of the *CAD* gene alone led to only small amounts of itaconate production. Four strategies have been successfully used to improve itaconic acid production: (i) enhancement of transporter systems (37, 38), including the mitochondrial aconitate transporter MttA (14) and a putative membrane major facilitator superfamily protein, MfsA; (ii) mitochondrial targeting of key enzymes (23); (iii) overexpression of aconitase (*aco*) (14, 23), which not only catalyzes citrate to form aconitic acid but also further transforms aconitic acid to isocitrate, necessitating improvement of the enzyme-substrate affinity of the three acids; (iv) increasing cytosol citrate formation through expression of the cytosolic citrate synthase gene *citB* (39). In this study, we reported a useful strategy for improving itaconate production through dynamic expression of the *CAD* gene during fermentation. The *sCAD* expression level of *Pgas-sCAD-2* increased gradually with the decrease in pH and finally reached a titer of around 5 g/liter, which was 5-fold higher than that of *PgpdA-sCAD-1*. Although NHEJ-mediated random integration was used for gene integration, itaconic acid production levels of either *PgpdA-sCAD* transformants or *Pgas-sCAD* transformants were nearly the same. Table 1 shows a comparison of the itaconate titers reported in the literature. Dynamic control has already been applied in *E. coli* (40) and *S. cerevisiae* (6) for the synthesis of products that are toxic to cells. In addition to metabolite concentrations (41, 42), other triggers, e.g., heat shock (43), quorum sensing (44), and stress response to intermediate toxicity (5), have served as tools for dynamic control. In this

TABLE 1 Itaconic acid titers of the engineered *A. niger* strains

<i>A. niger</i> strain	CAD location	CAD promoter	Coexpressed gene(s) or gene family(s)	Itaconic acid production (g/liter)	Culture method	Reference or source
AB 1.13 CAD <i>pyrG</i> — 10.1	Cytosol	<i>PgpdA</i>	— ^a	0.7	Flask	53
AB 1.13 CAD <i>pyr</i> + 4.1	Cytosol	<i>PgpdA</i>	<i>pyr</i>	1.945	Controlled batch cultivation	24
AB 1.13 CAD + MTT 1.4	Cytosol	<i>PgpdA</i>	MTT	1.5	Controlled batch fermentations	38
AB 1.13 CAD + MFS 3.9	Cytosol	<i>PgpdA</i>	MFS	1.5	Controlled batch fermentations	38
AB 1.13 CAD + MTT + MFS ₃	Cytosol	<i>PgpdA</i>	MTT, MFS	0.9	Controlled batch fermentations	38
AB 1.13 CAD + MTT + MFS #49	Cytosol	<i>PgpdA</i>	MTT, MFS	11.74	Controlled batch cultivation	39
AB 1.13 CAD + MFS + MTT + CitB	Cytosol	<i>PgpdA</i>	MFS, MTT, CitB	26.2	Controlled batch cultivation	39
ATCC 1015 cCAD	Cytosol	<i>Pmbf</i>	—	0.05	Flask	49
ATCC 1015 mCAD	Mitochondrion	<i>PicdA</i>	—	0.165	Flask	49
ATCC 1015 mCAD + mAcoA	Mitochondrion	<i>PicdA</i>	mACO	0.8	Flask	49
H915-1 <i>PgpdA</i> -sCAD	Mitochondrion	<i>PgpdA</i>	—	0.82	Flask	This study
H915-1 <i>Pgas</i> -sCAD	Mitochondrion	<i>Pgas</i>	—	4.92	Flask	This study

^a—, no additional genes are expressed.

study, a low-pH response promoter was used for dynamic control in *A. niger* for the first time, expanding available genetic tools for both academic and industrial applications.

The pH-mediated regulation of gene expression has been investigated in *A. nidulans*. The PacC signal transduction pathway is involved in the hyphal response under both acidic and alkaline growth conditions. PacC is a Zn-finger transcription factor that is activated through protein-protein interactions in a conserved signaling cascade composed of six *pal* genes (*palA*, *palB*, *palC*, *palF*, *palH*, and *palI*) (45) and Vps32 (46). PacC controls the expression of many functional genes at pH 5.0, including *gela* (45), a member of the Gas family of proteins, in *A. niger*. In addition, the PacC consensus binding sequence 5'-GCCARG-3' is found in *Pgas* at bp 916 to 928, indicating that the promoter may be bound by PacC. Nevertheless, the *Pgas* in this work was from *GelD*, whose regulator had not yet been reported, and the DNA pulldown results showed that PacC did not bind specifically to *Pgas* at pH 2.0 relative to the binding observed at pH 7.0, indicating that PacC was not a decisive factor in the control of *Pgas* expression and that other specific regulators may exist.

The protein Gas is a cell wall protein involved in the cell wall integrity (CWI) signaling pathway (47). There are five membrane sensor proteins in this pathway, along with GDP/GTP exchange factors, a small G-protein, mitogen-activated protein kinase (MAPK), MAPKK, MAPKKK, and the transcriptional factors Rlm1 and SBF (Swi4 and Swi6) (48). However, these transcription factors do not bind specifically with *Pgas* at pH 2.0. Notably, these two transcription factors were identified as *Pgas* regulators through DNA pulldown assays, and their specific localization in the nucleus at pH 2.0 but not at pH 7.0 supported their roles as positive regulators. Nevertheless, the roles of these regulators and the mechanisms of *Pgas* induction are still unclear, and future studies are needed to investigate the signal transduction pathway for *Pgas* regulation.

MATERIALS AND METHODS

Strains and media. *Escherichia coli* DH5 α was used as a host for recombinant DNA manipulation. *A. niger* H915-1, used as the parental strain for *A. niger* transformation, was provided by Jiangsu Guoxin Union Energy Co., Ltd. (Yixing, China), the third largest citrate producer in China.

E. coli transformants were grown in LB supplemented with 100 μ g/ml ampicillin. *A. niger* was cultured in ME medium (3% malt extract, 0.5% peptone) supplemented with 150 μ g/ml hygromycin. LBL medium contained 10 g/liter peptone and 5 g/liter yeast extract and was adjusted to pH levels between 2.0 and 7.0. Synthetic medium was used for itaconate fermentation, with an initial pH of 3.1 (24).

Selection of acid-inducible promoters of *A. niger* via microarray expression data. The Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo/>) was used to retrieve gene expression data (accession number GSE11725), which were based on the Affymetrix chip platform (GPL5975) (16). Loci exhibiting changes in expression between pH 4.5 and pH 2.5 were identified.

Mycelia for RNA sequencing were sampled at 6, 12, 24, and 48 h during citrate fermentation and the protein corresponding to GEO accession number GSE74544 was identified. Genes with increased expression levels at low pH with the protein GSE11725 were further analyzed using the expression data regarding the protein GSE74544. Genes that were upregulated in both data sets with high FPKM values were selected as acid inducible, and the 1.5-kb fragment located upstream of the coding sequence was

TABLE 2 Plasmids and *A. niger* strains used in this study

Strains or plasmid	Relevant genotype or characteristics	Function	Source
<i>A. niger</i> strains			
WT	H915-1, an industrial citrate-producing strain, host for gene expression		Jiangsu Guoxin Union Energy Co., Ltd.
PgpdA-sGFP	<i>PgpdA::sGFP</i>		This study
Pgas-sGFP	<i>Pgas::sGFP</i>		This study
PgpdA-sCAD	<i>PgpdA::sCAD</i>		This study
Pgas-sCAD	<i>Pgas::sCAD</i>		This study
Plasmids			
pMHT	<i>Pmbf::hph::Ttrp</i>	Construction of strains PgpdA-sGFP and Pgas-sGFP	
pGGT	<i>PgpdA::sGFP::Ttrp</i>	Construction of strain PgpdA-sGFP	
pGASGT	<i>Pgas::sGFP::Ttrp</i>	Construction of strain Pgas-sGFP	
pHGSCAD	<i>pPmbf::hph::Ttrp PgpdA::sCAD::Ttrp</i>	Construction of strain PgpdA-sCAD	
pHASCAD	<i>pPmbf::hph::Ttrp Pgas::sCAD::Ttrp</i>	Construction of strain Pgas-sCAD	

analyzed using Neural Network Promoter Prediction software (version 2.2; http://www.fruitfly.org/seq_tools/promoter.html). This fragment was amplified from the genome of *A. niger* H915-1 and was used as the promoter sequence in this work.

Construction of expression vectors. Molecular cloning techniques were performed using standard procedures. The hygromycin resistance vector pMHT contained the *mbf* promoter amplified from the *A. niger* H915-1 genome (49), the *hph* gene from pRS303 (GenBank accession number [U03435.1](#)), and the Trp terminator (GenBank accession number [X02390.1](#)), amplified from the *A. nidulans* genome. These fragments were joined together through fusion PCR and connected to a pMD19-T simple vector (TaKaRa, Tokyo, Japan) to generate pMHT.

For construction of a GFP expression plasmid, the *gpdA* (GenBank accession number [Z32524](#)) promoter was amplified from the *A. nidulans* genome, sGFP was synthesized with optimal human codons (50), and both constructs were connected to pUC18 (GenBank accession number [L08752.1](#)) with Ttrp. The 1.5-kb *gas* (EuPathDB identification number [An09g00670](#)) promoter was amplified from the *A. niger* H915-1 genome and connected to the pMD19-T vector (TaKaRa) by using sGFP and Ttrp.

To construct *cis*-aconitate decarboxylase (*CAD*) expression cassettes, the *CAD* coding sequence was amplified by reverse transcription-PCR (RT-PCR) from *A. terreus* HAT418 (the protein sequence was the same as that reported for GenBank accession number [BAG49047.1](#)). The 24-amino-acid mitochondrial signal peptide of the aconitase gene from *A. niger* (GenBank accession number [EHA18500.1](#)) was synthesized and added to the 5' end of the *CAD* gene (designated *sCAD*). The plasmid backbone was the pMD19 simple vector.

All plasmids and strains used in this study are listed in Table 2. Primers are listed in Table 3, the genes analyzed are summarized in Table 4, and construction details are illustrated in Fig. S1 in the supplemental material.

Transformation of *A. niger* H915-1. The polyethylene glycol (PEG)-mediated transformation of protoplasts was performed based on a method published by Blumhoff et al. (49). All transformants were purified three times via single-colony isolation on selection medium. Transformants were verified by PCR using specific genomic primers.

Gene copy number determinations. The copy numbers of transgenes were estimated by real-time qPCR (51). Actin was selected as a single-copy control gene. Primers for qPCR were designed using the Beacon Designer (Table 3) with product lengths between 120 and 150 bp and primer melting temperature (T_m) values of 55°C. qPCR was performed using a LightCycler 480 apparatus (Roche Applied Science, Indianapolis, IN, USA) with SYBR premix ExTaqII (TaKaRa). Strains with a single copy of the transformed gene were used for further analysis.

sGFP detection by fluorescence microscopy. Conidia (3×10^5 /ml) of *A. niger* transformants were inoculated in LBL medium at pH 5.0 and cultured at 35°C with shaking at 120 rpm for 16 h. Samples were then washed twice with distilled water, and mycelia were treated in LBL with different pH values for 4 h. After washing with distilled water, the samples were observed under a microscope, using blue light to detect the fluorescent of sGFP.

Relative sGFP fluorescence strength. Conidia (3×10^5 /ml) of *A. niger* transformants were inoculated in ME medium and cultured at 35°C for 24 h. Conidia were washed twice with distilled water, and the pellets were treated in LBL with different pHs for 4 h. The pellets were harvested, washed twice with WS buffer (100 mM Tris, pH 7.0), and dried with filter paper immediately. The pellets were transferred to MP lysing matrix C (MP Biomedicals, Heidelberg, Germany), and the mycelia were disrupted with six cycles of 30 s at 5 m/s using a FastPrep-24 (MP Biomedicals, New York, NY, USA). After centrifugation at $14,000 \times g$ and 4°C for 10 min, the supernatant was obtained as a protein sample. The total protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). The protein concentration of each sample was diluted to 50 µg/ml, and the exact protein concentration was determined (assigned the letter A). A Cytation 3 cell imaging multimode reader was used to detect fluorescence, with an excitation wavelength

TABLE 3 Primers used in this study

Primer use category and name	Sequence (5' → 3') ^a
Construction of plasmids and amplification of expression cassettes	
mbf-F	GTACAGTGGCCATGAAATCCAA
mbf-R-hph	TGAGTTCAGGCTTTTACCCATTTTGAAGATGGATGAGAAGTCGGTG
mbf-F-hph	CACCGACTTCTCATCCATCTTCAAAATGGGTAAAAAGCCTGAACTCA
hph-R-trpC	TTTGATGATTTAGTAACGTTAAGTGGATCTTATCCTTTGCCCTCGGACGAGT
hph-F-trpC	ACTCGTCCGAGGGCAAGGAATAAGATCCACTTAACGTTACTGAAATCATCAA
trpC-R	TCAAGTGGAGATGTGGAGTGGGCGCTTA
gpd-F	<u>CCCGGGCAATTCCCTTGTATCTCTACACACAG</u>
gpd-R	<u>GGATCCGGTGATGTCTGCTCAAGCGG</u>
trp-F	<u>CTGCAGAGATCCACTTAAACGTTACTGAAATC</u>
trp-R	<u>AAGCTTCTCGAGTGGAGATGTGGAGTGG</u>
gas-F	<u>GAATTCCTGCTCTCTCTGCTCTCTTTCT</u>
gas-R	<u>CCCGGGGTGAGGAGGTGAACGAAAGAAGAC</u>
GFP-F1	GATCCATGGTGAGCAAGG
Ttrp-R	<u>AAGCTTTCGAGTGGAGATGTGGAGTGG</u>
51-gpd-F2	<u>GAATTCGCGGCCGCCAATCCCTTGTATCTCTACACACAG</u>
51-gpd-R2	<u>GGTACCGGTGATGTCTGCTCAAGCGG</u>
51-trp-R2	<u>AAGCTTACTAGTCTCGAGTGGAGATGTGGAGTGG</u>
51-trp-F4	<u>GGATCCGATCCACTTAAACGTTACTGAAATC</u>
51-CAD-sig-F1	TGGGTGCGCTGGCCCTAAGTCCCGCTTATGCTCGGGGCTCGGGGTATGACCAACAATCTGCGGACA
51-CAD-sig-F2	<u>CGGGTACC</u> ATGATTACCACAAGGCTTGC GCGCATGGGTGCGCTGGCCCTAA
61-CAD-R2	<u>CGGGATCCTTATACCA</u> GTGGCGATTTACG
61-CAD-F2	<u>CGGGTACC</u> ATGACCAACAATCTGCGGACA
1-mbfEnz-F	GTACAGTGGCCATGAAATCCA
1-trpXEnz-R	<u>AGACTAGTCTAGAAGGAAAAAGCGCCGCTCTAGAAAGAAGGATTACCTCTAAAC</u>
71-gas-F2	<u>GAATTCGCGGCCGCTGCTCTCTCTGCTCTCTTTCT</u>
71-gas-R2	<u>GGTACCGTGAGGAGGTGAACGAAAGAAGAC</u>
gas-sm-F	CGGGTCTCGGATGCAGCCGGCTTGG
gas-sm-R	CCAAGCCGGTGTCATCCGAGACCCG
pMD-F	GGCCATGAAATCCAATCATTTT
pMD-R	GAGTGGGCGCTTACACAGTA
qPCR	
actin-F	CGGAACCTATTGGCTTGG
actin-R	ACAGAACGATATTGAGGTAGA
GFP-q-F	CGCCGAGGTGAAGTT
GFP-q-R	GTGGCTGTTGTAGTTGTAC
Hph-F	GATATGTCCTGCGGGTAA
Hph-R	CCAATGTCAAGCACTTCC
CAD-2-F	GATCCATGGTGAGCAAGG
CAD-2-R	TGAGTTGGCTCTATTCAA
Synthesis of DNA pulldown probe	
Gas-2-F(biotin)	CTGCTCTCTCTGCTCTCTTT
Gas-2-R	CTTCTTTCGTTACCTCTCAC

^aRestriction sites for the endonuclease are underlined.

of 485 nm and emission wavelength of 535 nm, and the fluorescence intensity was labeled B. The standardized fluorescent intensity was estimated as B divided by A.

Shake flask cultivation for itaconate fermentation. The conidia concentration was determined by counting dilution factors using a hemocytometer. Conidia at a concentration of 10⁶/ml were inoculated

TABLE 4 Genes analyzed in this study

Gene name	Protein ID ^a of gene product in <i>A. niger</i> strain		Annotation
	CBS 513.88	H915-1	
<i>fnx</i>	An03g00680	evm.model.1.1208	Strong similarity to multidrug resistance protein Fnx1
<i>dhy</i>	An03g00670		Hypothetical dehydrogenase
<i>gas</i>	An09g00670	evm.model.unitig_5.1027	Strong similarity to 1,3-beta-glucanosyltransferase
<i>patl</i>	An16g01200	evm.model.unitig_6.853	Amino acid/polyamine transporter I
<i>amy</i>	An12g02460	evm.model.unitig_3.181	Hypothetical alpha-amylase
<i>pth</i>	An14g03530	evm.model.unitig_5.281	Hypothetical protein, contains 6 predicted transmembrane domains
<i>aat</i>	An01g01940	evm.model.unitig_2.972	Amino acid transporters

^aProtein ID numbers for both strains are from the NCBI database.

into seed medium and cultured for 24 h at 250 rpm at 35°C. With 10% inoculum, the fermentation was performed in 100 ml of culture medium at 250 rpm and 35°C for 6 days in a 500-ml flask. Each culture was carried out with at least three biological replicates.

HPLC-based detection of organic acids. For HPLC analysis, samples were collected and centrifuged at $14,000 \times g$ for 10 min at 4°C, and the supernatant was filtered with a 0.22- μ m filter membrane after dilution. HPLC was performed using an 87 H-Aminex organic acids column (Bio-Rad, Richmond, CA, USA), a photodiode array (PDA) detector, and a refractive index (RI) detector, with 5 mM H₂SO₄ as an eluent at a speed of 0.6 ml/min at 35°C.

qPCR to verify expression of genes. For verification of gene expression induced by low pH during itaconate fermentation, the samples were collected at 8, 24, 48, 84, and 108 h. For analysis of *Pgas* induction conditions, conidia from sCAD transformants (10⁶/ml) were culture in ME at 35°C for 24 h and harvested using a Miracloth. The pellets were washed with distilled water and incubated in certain induction medium for 4 h.

After sampling, the hyphal pellets were quickly washed with distilled water and frozen in liquid nitrogen immediately. Total RNA was isolated using an RNeasy plant minikit (Qiagen, Germantown, MD, USA). PrimeScript RT reagent kit with gDNA Eraser (TaKaRa) was used to prepare cDNA. Primers for qPCR are listed in Table 3 and had product lengths between 120 and 150 bp and primer *T_m* values of 55°C. Actin was selected as a reference gene. Error bars represent the standard deviations from three biological replicates.

DNA pulldown. Conidia (3×10^5 /ml) of *Pgas*-sCAD-2 were inoculated in ME medium and cultured at 35°C for 24 h. Samples were washed twice with distilled water, and the pellets were treated in LBL at pH 2.0 or 7.0 for 4 h. The pellets were harvested, washed twice with distilled water, dried with filter paper, and treated immediately with a fungal nuclear protein extraction kit (BestBio Corp., Shanghai, China) following the manufacturer's instructions. Protein concentrations in the nuclear extracts were determined with a BCA protein assay kit (Beyotime, Jiangsu, China). After equilibration with wash buffer (150 mM NaCl, 30 mM Tris-Cl, 10% glycerol; pH 7.5), Sefinose resin was used to separate nuclear proteins without nonspecific binding, and the flowthrough was adjusted to the same concentration for subsequent steps. The biotin-labeled *Pgas* probe was obtained through PCR with biotin-labeled primers synthesized by Genewiz (Suzhou, China). The primer sequences are shown in Table 3. The PCR products were purified using a gel extraction kit (Sangon Biotech, Shanghai, China). The 20-bp control probe was obtained through annealing of the biotin-labeled random primers with their complementary sequences, which were also synthesized by Genewiz. Each type of probe was added to wash buffer-equilibrated streptavidin-agarose resin (Pierce, Rockford, IL, USA). After slow mixing using a vortex for 30 min at 4°C, the *Pgas* probe was bound to streptavidin-agarose. Subsequently, 4 ml of nuclear protein was added and gently mixed with the probe binding streptavidin-agarose for 4 h at 4°C. The resin was centrifuged at $800 \times g$ for 1 min, washed twice with wash buffer, and added to 2 $\times$ sodium dodecyl sulfate (SDS) sample buffer for SDS-polyacrylamide gel electrophoresis (PAGE). The polyacrylamide gel was stained with silver stain (52), and the protein bands were analyzed using shotgun LC-MS/MS (LCQ Deca XP; Thermo-Finnigan, San Jose, CA, USA).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.03222-16>.

TEXT S1, PDF file, 0.7 MB.

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X.Y. designed and performed the experiments. J.L., H.-D.S., L.L., G.D., and J.C. conceived the project, analyzed the data, and wrote the paper.

We declare no competing financial interests.

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