The G-Protein α-Subunit GasC Plays a Major Role in Germination in the Dimorphic Fungus *Penicillium marneffei*

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Manuscript received December 8, 2002
Accepted for publication March 3, 2003

ABSTRACT

The opportunistic human pathogen *Penicillium marneffei* exhibits a temperature-dependent dimorphic switch. At 25°, multinucleate, septate hyphae that can undergo differentiation to produce asexual spores (conidia) are produced. At 37° hyphae undergo arthroconidiation to produce uninucleate yeast cells that divide by fission. This work describes the cloning of the *P. marneffei gasC* gene encoding a G-protein α-subunit that shows high homology to members of the class III fungal Gα-subunits. Characterization of a Δ*gasC* mutant and strains carrying a dominant-activating $gasC^{G45R}$ or a dominant-interfering $gasC^{G207R}$ allele show that GasC is a crucial regulator of germination. A Δ*gasC* mutant is severely delayed in germination, whereas strains carrying a dominant-activating $gasC^{G45R}$ allele show a significantly accelerated germination rate. Additionally, GasC signaling positively affects the production of the red pigment by *P. marneffei* at 25° and negatively affects the onset of conidiation and the conidial yield, showing that GasC function overlaps with functions of the previously described Gα-subunit GasA. In contrast to the *S. cerevisiae* ortholog Gpa2, our data indicate that GasC is not involved in carbon or nitrogen source sensing and plays no major role in either hyphal or yeast growth or in the switch between these two forms.

MOST fungal infections in both animals and plants are initiated by contact of the host with spores, which begin the infective process by undergoing germination. The early molecular events involved in sensing and transmitting the signal to germinate are not well understood, but represent a key issue in understanding the early steps of fungal infections (d'Enfert 1997; Osherov and May 2001). The opportunistic human pathogen *Penicillium marneffei* is a dimorphic ascomycete that exhibits a temperature-dependent dimorphic switch (Segretain 1959; Garrison and Boyd 1973; Andrianopoulos 2002). After germination of the asexual spore (conidium) at 25°, multinucleate, septate hyphae are produced by apical growth and lateral branching. Exposure of hyphae to an air interface induces asexual development at 25°, producing conidia borne on specialized multicellular structures called conidiophores. The conidia represent the primary means for dispersal. At 37°, such as after inhalation by a host, conidia germinate to produce hyphae that undergo a process known as arthroconidiation, in which septation and nuclear division become coupled. The hyphae lay down double septa and, following cell separation, produce single uninucleate yeast cells that divide by fission and disseminate throughout the body (Chan and Chow 1990; Cooper and McGinnis 1997; Vossler 2001).

Heterotrimeric guanine nucleotide-binding proteins (G-proteins) act as signal transducers that couple cell surface receptors to cytoplasmic effector proteins and are conserved in all eukaryotes. The G-protein-coupled receptors (GPCR) sense various agonists such as photons, odorants, neurotransmitters, pheromones, and sugars. Upon activation of the Gα-subunit triggered by conformational change of the receptor, GDP is exchanged for GTP and the Gα-subunit dissociates from the Gβγ complex. Both Gα- and Gβγ-subunits are able to trigger downstream signaling pathways by interacting with various targets such as phosphodiesterases, protein kinases, adenylyl cyclases, phospholipases, and ion channels (Kaziro *et al.* 1991; Simon *et al.* 1991; Neer 1995; Hamm and Gilchrist 1996).

In fungi, three different classes of Gα-subunits have been identified. The class III fungal Gα-subunits are particularly interesting, as many members play crucial roles in the regulation of various morphological and developmental processes as well as pathogenicity (Bölker 1998; Borges-Walmsley and Walmsley 2000).

Most of our knowledge about class III fungal Gα-subunits comes from studies of the morphological switch from yeast to pseudohyphal growth in the model organism *Saccharomyces cerevisiae*. The Gα-subunit Gpa2 in *S. cerevisiae* is required for the induction of pseudohyphal growth in diploid yeast cells in response to nitrogen starvation in the presence of an abundant fermentable carbon source such as glucose. The mechanism by which Gpa2 is involved in transducing the nitrogen starvation signal has not yet been defined (Kübler *et al.* 1997;

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession no. AY170625.

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Lorenz and Heitman 1997; Lorenz *et al.* 2000). However, Gpa2 has been shown to interact with the GPCR Gpr1 whose expression is upregulated during nitrogen starvation (Xue *et al.* 1998). Gpr1 has been proposed to be a glucose receptor. When glucose is added to a yeast culture previously starved for glucose, a rapid and transient increase in cyclic adenosine 3′, 5′-monophosphate (cAMP) is observed. This response is impaired in both Δ*gpa2* and Δ*gpr1* mutants and a dominant-activating *GPA2* allele bypasses the need for high extracellular glucose concentrations normally required to activate the Gpr1-Gpa2 system (Colombo *et al.* 1998; Kraakman *et al.* 1999; Rolland *et al.* 2000). The addition of exogenous cAMP restores the pseudohyphal growth defect observed in both Δ*gpa2* and Δ*gpr1* mutants and is evidence of Gpa2 signaling through the cAMP-dependent protein kinase A (cAMP-PKA) pathway (Kübler *et al.* 1997; Lorenz and Heitman 1997).

In *S. cerevisiae*, the regulation of pseudohyphal growth is regulated not only by the Gpr1-Gpa2 system, but also by Ras2, which signals both to the cAMP-PKA pathway and to protein kinases involved in mitogen-activated protein kinase signaling, such as Ste20, Ste11, and Ste7 (Toda *et al.* 1985; Moesch *et al.* 1996). Strains carrying a dominant-activating *RAS2* allele show greatly enhanced pseudohyphal growth (Gimeno *et al.* 1992). Interestingly, Ras2 also plays a crucial role in ascospore germination. Spores harboring a Δ*ras2* mutation show a delay in germination, whereas overexpression of the *RAS2* gene leads to an increased rate of germination (Herman and Rine 1997). The role of the Gpr1-Gpa2 system in germination has not been investigated.

Git3 and Gpa2 in *Schizosaccharomyces pombe* are orthologs of Gpr1 and Gpa2 in *S. cerevisiae*. As in *S. cerevisiae*, the Git3-Gpa2 system responds to glucose and subsequently stimulates the cAMP-PKA pathway (Isshiki *et al.* 1992; Welton and Hoffman 2000). This signaling pathway is crucial for the efficient initiation of spore germination as spores harboring mutations in *gpa2*, *cyr1* (encoding for adenylate cyclase), or *pka1* (encoding for PKA) are severely impaired in germination (Hatanaka and Shimoda 2001). Additionally, the Git3-Gpa2 system is also required for mating in response to nitrogen starvation (Isshiki *et al.* 1992).

In the filamentous fungus *Aspergillus nidulans*, the taxonomically closest model fungus to *P. marneffei*, a cAMP-PKA pathway has recently been shown to be required for efficient germination of conidia. The presence of water and a carbon source such as glucose is sufficient to activate a conidium and trigger germination. During this early commitment step trehalose is rapidly mobilized before any morphological changes are evident. This is followed by an isotropic growth stage (swelling) involving water uptake and cell wall growth, and eventually wall deposition becomes polarized, resulting in the formation of a germ tube (d'Enfert 1997; Osherov and May 2001). Deletion of the *A. nidulans* gene encoding PKA (*pka*A) results in delayed germ tube emergence while deletion of the gene encoding adenylate cyclase (*cyaA*) makes this delay even more pronounced (Fillinger *et al.* 2002). Similar to the role of *S. cerevisiae* Ras2 in ascospore germination, *A. nidulans* strains overproducing a dominant-activating form of RasA produce giant swollen conidia with multiple nuclei that fail to produce germ tubes. RasA has also been suggested to mediate carbon source sensing during the germination process (Som and Kolaparthi 1994; Osherov and May 2000).

FadA, the only Gα-subunit that has been hitherto characterized in *A. nidulans*, is a key regulator of conidiation and the production of secondary metabolites. A strain carrying a dominant-activating $fadA^{G42R}$ allele is aconidial and does not produce the mycotoxin sterigmatocystin (ST; Yu *et al.* 1996; Hicks *et al.* 1997). Interestingly, conidiation is partially restored in a Δ*pkaA* $fadA^{G42R}$ double mutant, showing that PKA activity in *A. nidulans* inhibits conidiation as well as ST production (Shimizu and Keller 2001). Although this suggests that FadA signaling is partially mediated by PkaA, the data also show that FadA signals in a PkaA-independent fashion (Shimizu and Keller 2001).

We have previously reported that the *P. marneffei* Gα-subunit GasA, an ortholog of *A. nidulans* FadA, is a key regulator of conidiation. Strains carrying a dominant-activating $gasA^{G42R}$ allele are locked in vegetative growth and are therefore aconidial, while strains carrying the dominant-interfering $gasA^{G203R}$ allele show inappropriate conidiation. GasA is also a regulator of secondary metabolites, but is not involved in dimorphic switching or yeast growth at 37° (Zuber *et al.* 2002). This work describes the cloning of the *P. marneffei gasC* gene encoding a G-protein α-subunit that shows high homology to members of the class III fungal Gα-subunits. Characterization of a Δ*gasC* mutant and strains carrying a dominant-activating $gasC^{G45R}$ or a dominant-interfering $gasC^{G207R}$ allele show that GasC is a crucial regulator of germination. This is the first report showing that a Gα-subunit is involved in germination in filamentous fungi. Furthermore, GasC is a regulator of secondary metabolites and, to a lesser extent, a regulator of conidiation, showing that GasC function overlaps with that of GasA. In contrast to Gpa2 in *S. cerevisiae*, our data indicate that GasC is not involved in carbon or nitrogen source sensing. Moreover, GasC plays no major role in either hyphal or yeast growth or in the switch between these two forms.

MATERIALS AND METHODS

Fungal strains, media, and growth conditions: Fungal strains used in this study and their genotypes are listed in Table 1. Transformation of the *P. marneffei* strain SPM4 and the *A. nidulans* strain A770 was performed as previously described (Andrianopoulos and Hynes 1988; Borneman *et al.* 2001). The Δ*gasC* mutant strain (TS32-7-8) was generated by transformation of SPM4 with 500 ng of a gel-purified *Not*I/*Xho*I fragment from pSZ5103 and selection for $pyrG^{+}$. SPM4 was transformed with the appropriate plasmids to create strains carrying

the dominant-interfering $gasC^{G207R}$, the dominant-activating $gasC^{G45R}$, the wild-type *gasC*, the double dominant-interfering [$gasA^{G203R}gasC^{G207R}$], and the double dominant-activating [$gasA^{G42R}$ $gasC^{G45R}$] alleles. The *A. nidulans* strain A770 was transformed with the appropriate plasmids to create strains carrying the dominant-interfering $gasC^{G207R}$, the dominant-activating $gasC^{G45R}$, and the wild-type *gasC* allele.

At 25°, *P. marneffei* strains were grown on Aspergillus nitrogen-free medium (ANM) or ANM without any carbon source (CF) and supplemented with 10 mM γ-amino butyric acid (GABA) or 10 mM ammonium sulfate [$(NH_4)_2SO_4$] as a nitrogen source (Cove 1966). Glucose as a carbon source was used at concentrations of 1 or 0.1% (w/v); ethanol as a carbon source was used at a concentration of 1% (v/v). Where appropriate, the media were supplemented with 0.3 M NaCl, 0.3 M KCl, 0.5 M sorbitol, or 10 mM theophylline + 0.1 mM dibutyryl-cAMP (dbcAMP). At 37°, *P. marneffei* strains were grown on *S. cerevisiae* synthetic dextrose (SD) medium or on brain heart infusion (BHI) broth (Oxoid; Ausubel *et al.* 1994). *A. nidulans* strains were grown at 37° on ANM supplemented with 10 mM ammonium tartrate (NH_4T) or 10 mM ammonium sulfate [$(NH_4)_2SO_4$] as a nitrogen source. Glucose as a carbon source was used at concentrations of 1 or 0.1% (w/v). Where appropriate, the media were supplemented with 1 M NaCl. Strains SPM4 and A770 were grown in the presence of 10 mM uracil and, where appropriate, transformants were tested on media containing 10 mM uracil to confirm that the observed phenotypes were unrelated to poor expression of the *pyrG* selectable marker. The growth conditions used for the preparation of *P. marneffei* for RNA extractions have been described previously (Borneman *et al.* 2001).

Molecular techniques: Plasmid DNA was isolated using the high-purity plasmid kit (Roche Diagnostics). Genomic DNA from *P. marneffei* was isolated as previously described (Borneman *et al.* 2001). RNA was prepared using the FastRNA kit (Bio101, Vista, CA) as previously described (Borneman *et al.* 2001). Southern and Northern blotting was performed with Amersham Hybond N+ membranes according to the manufacturer's instructions. Filters were hybridized using [α-^{32}P]dATP-labeled probes by standard methods (Sambrook *et al.* 1998). As a loading control Northern blots were probed with a histone H3 gene probe (Ehinger *et al.* 1990).

Degenerate PCR was performed on genomic DNA of strain FRR2161 using the sense primer FENVT (5′ GCTTCGAGAAC GTGACCTCCRTNATHTTYTG 3′) and the antisense primer KETIL (5′ CAGGGCGTTCTGCAGGATNGTYTCYTT 3′), corresponding to highly conserved regions specific to the fungal Gα III class. Primers were designed using the consensus-degenerate hybrid oligonucleotide primers (CODEHOP) method (Rose *et al.* 1998). PCR conditions consisted of a hot start with cycling parameters of 94° for 30 sec, 45° for 30 sec, and 72° for 1 min, with 35 cycles in a Mastercycler gradient thermocycler (Eppendorf, Madison WI). The products were cloned into pGEMTeasy (Promega, Madison, WI). A 550-bp insert with high homology to several members of the fungal Gα III class was used to probe a 7- to 8-kb *Sac*II/*Bgl*II size-selected FRR2161 genomic library in pBluescript II SK+ (Stratagene, La Jolla, CA). Sequencing of a positive clone (pSZ4928) revealed that it contained the entire coding region, but only 194 bp of 5′ untranslated region. Therefore, a 3- to 4-kb *Pst*I/*Hin*dIII size-selected FRR2161 genomic library in pBluescript II SK+ was probed and a second positive clone (pSZ4977) was isolated. A 2.4-kb *Hin*dIII fragment of pSZ4928 was cloned into pSZ4977 linearized with *Hin*dIII to create a full-length *gasC* clone (pSZ5038). Sequencing was performed by the Australian Genome Research Facility and analyzed with Sequencher 3.1.1 (Gene Codes, Ann Arbor, MI). The GenBank accession number of the *P. marneffei gasC* gene is AY170625. Database searches and sequence comparisons were performed using the Australian National Genomic Information Service. Sequence alignments were performed using Eclustalw (GCG software package; Thompson *et al.* 1994) and MacBoxShade sequence analysis tools.

To disrupt *gasC*, a derivative of pSZ5038 containing a 2.1-kb *Pst*I/*Hin*dIII fragment downstream of *gasC* was digested with *Spe*I and *Pst*I and a 2.2-kb *Spe*I/*Pst*I fragment containing the *pyrG* cassette of pAB4626 was inserted (Borneman *et al.* 2002). This plasmid was digested with *Spe*I and *Sma*I and a 2-kb *Spe*I/*Sal*I end-filled fragment of pSZ4977 containing sequences upstream of *gasC* was inserted, generating the knockout plasmid pSZ5103.

The dominant-activating $gasC^{G45R}$ and the dominant-interfering $gasC^{G207R}$ alleles were created by inverse PCR (McPherson *et al.* 1991) using gasC.dom.up (5′ CGGGAAAGTGGAAAGT CAACC 3′; 770–790; base mismatches are underlined), gasC.dom.lo (5′ GGAGCCTGTAGATTAGATAAAACAAG 3′; 744–769), gasC.neg.up (5′ CGTCAACGCAGTGAACGAAAGAAATG 3′; 1357–1382; base mismatches are underlined), and gasC.neg.lo (5′ GCCGACATCAAACATGCTATAAC 3′; 1334–1356), respectively. The plasmids pSZ5112 ($gasC^{G45R}$) and pSZ5113 ($gasC^{G207R}$) were sequenced and used to recreate full-length *gasC* clones, pSZ5114 and pSZ5115. A 4.1-kb *Xba*I/*Bam*HI fragment of pSZ5114 and pSZ5115 was cloned into pALX223 digested with *Xba*I and *Bam*HI to generate pSZ5135 ($gasC^{G45R}$) and pSZ5136 ($gasC^{G207R}$), respectively. The plasmid pALX223 contains the *pyrG* gene for direct selection in the *P. marneffei* SPM4 strain and the *A. nidulans* A770 strain (A. Andrianopoulos, unpublished data). Additionally, the full-length wild-type *gasC* allele was cloned into pALX223, generating pSZ5134. The plasmid carrying the double dominant-activating [$gasA^{G42R}gasC^{G45R}$] alleles (pSZ5447) was created by inserting a 4-kb *Xba*I/*Bam*HI fragment of pSZ5114 ($gasC^{G45R}$) into pSZ5030 ($gasA^{G42R}$; Zuber *et al.* 2002) digested with *Xba* and *Bam*HI. The plasmid carrying the double dominant-interfering $gasA^{G203R}gasC^{G207R}$ alleles (pSZ5448) was created by inserting a 4-kb *Xba*I/*Bam*HI fragment of pSZ5115 ($gasC^{G207R}$) into pSZ5089 ($gasA^{G203R}$; Zuber *et al.* 2002) digested with *Xba*I and *Bam*HI.

Quantitation of conidial yields: Conidia (1×10^5) of each strain were spread on 1% ANM + GABA, 1% ANM + GABA + 0.3 M NaCl, 0.1% ANM + GABA, and 0.1% ANM + $(NH_4)_2SO_4$ solid medium and incubated at 25° for 8 days. Conidia from a defined area (2.5 cm^2) were harvested and an appropriate dilution counted with a hemocytometer.

Germination experiments: Conidia (2×10^6) of each strain were inoculated into 300 μl of 0.1% ANM + $(NH_4)_2SO_4$, CF + EtOH + $(NH_4)_2SO_4$, or CF + $(NH_4)_2SO_4$ liquid medium and incubated at 25° in a 24-well microtiter plate without shaking. Germlings were viewed on an inverted Olympus IX70 microscope at the time points specified. Images were captured digitally using a Photometrics Coolsnap fx camera at the appropriate time points and processed using IPlab (Scanalytics) and Adobe Photoshop software.

Microscopy: *P. marneffei* strains were grown on slides covered with thin layers of solid medium and resting in liquid medium (Borneman *et al.* 2000). Slides were mounted with the addition of 500 μg/μl of 4′,6-diamidino-2-phenylindole (DAPI) and viewed using either differential interference contrast (DIC) or epifluorescence optics on a Reichart Jung Polyvar II microscope. Images were captured digitally using a SPOT CCD camera (Diagnostic Instruments) and processed using Adobe Photoshop software.

RESULTS

***P. marneffei* GasC is related to class III fungal Gα-proteins:** A gene encoding a Gα-subunit from *P. marneffei* was

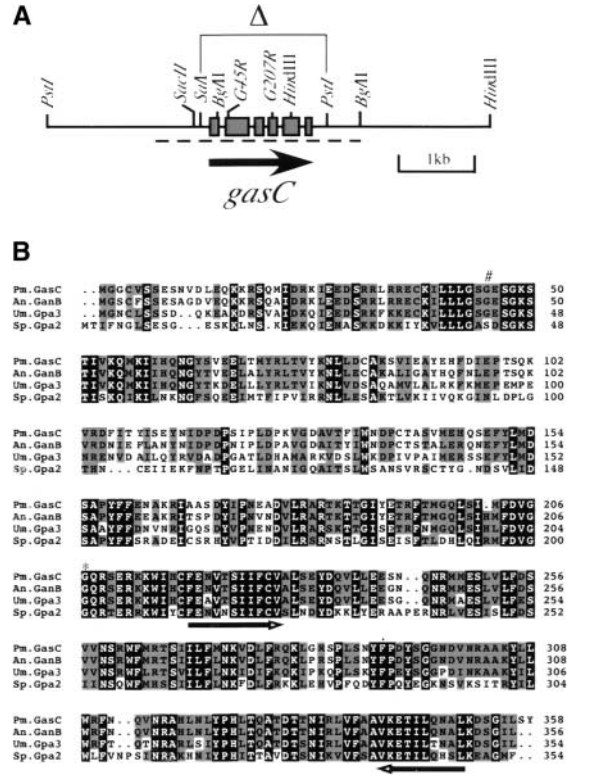

FIGURE 1.—The *gasC* gene of *P. marneffei* encodes a Gα-subunit of a heterotrimeric G-protein related to class III fungal Gα-proteins. (A) Gene structure and partial restriction map of the *gasC* gene corresponding to pSZ5038. The region predicted to encode the GasC protein is indicated by shaded boxes representing the exons and interrupted by five introns. The solid arrow shows the direction of transcription. The dashed line represents the sequenced part of the clone. The sequence deleted in the Δ*gasC* mutant is indicated. (B) Alignment of the deduced amino acid sequence of *P. marneffei* GasC with *A. nidulans* GanB (An.GanB; AF198116), *U. maydis* Gpa3 (Um.Gpa3; U85777), and *S. pombe* Gpa2 (Sp.Gpa2; D13366). The dominant-activating (G45R) and the dominant-interfering (G207R) mutations described in the text are indicated by # and *, respectively. The conserved regions highly specific to class III fungal Gα-subunits, indicated by arrows, were used to design degenerate primers for PCR. Identical (solid background) and similar (shaded background) amino acids are marked.

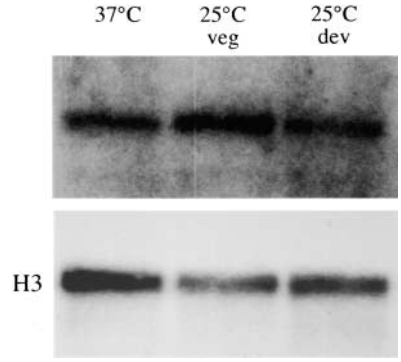

FIGURE 2.—Northern blot analysis of *gasC* expression. Total RNA from *P. marneffei* wild type was isolated from yeast cultures (37°), vegetative mycelia grown in liquid at 25° (25° veg), and asexually developing cultures (25° dev). RNA from each of the three growth stages (37°, 25° veg, and 25° dev) was hybridized with probes specific for either *gasC* or histone H3 (H3). Histone H3 was used as a loading control.

isolated using a degenerate primer PCR-based approach with primers specific to class III fungal Gα-proteins. Sequence analysis of the 550-bp PCR product showed significant homology to members of the fungal Gα III class (BÖLKER 1998). Southern blot analysis using the amplification product as a probe indicated a gene present in single copy in *P. marneffei* (data not shown). This gene was designated *gasC* (*G*α-subunit) and the amplification product was used to screen a partial genomic DNA library. The clone containing the entire *gasC* gene was sequenced and is predicted to contain five introns on the basis of comparison with closely related homologs (Figure 1A). The deduced GasC protein sequence of 358 amino acids shows 88.2% identity with the putative Gα-subunit encoded by the *A. fumigatus* gene *AFA5C11.9c* (accession no. AL713629) and 87.9% identity with the Gα-subunit GanB from *A. nidulans* (accession no. AF198116; Figure 1B). This shows that *P. marneffei* GasC is a member of the fungal Gα III class.

***gasC* is expressed during all growth stages:** RNA from wild-type *P. marneffei* was isolated from three growth and developmental stages: vegetative hyphal cells grown in liquid medium at 25°, conidiating (asexually developing) cultures at 25°, and yeast cells at 37°. Northern blot analysis using a 0.6-kb *gasC* fragment as a probe identified a single 1.4-kb transcript. The *gasC* transcript was readily detected and showed the same expression level in all three developmental stages, suggesting that *gasC* is constitutively expressed in *P. marneffei* (Figure 2).

Generation of *gasC* mutant alleles: To investigate the function of GasC, a number of mutant *gasC* alleles were generated. A knock-out plasmid (pSZ5103) in which the entire *gasC* coding sequence was replaced with the *A. nidulans pyrG* gene (Figure 1A) was used to transform the SPM4 strain and delete *gasC* by homologous gene replacement. A total of 39 transformants were isolated by direct selection for $pyrG^{+}$ and subjected to Southern blot analysis. A Δ*gasC* mutant in which the endogenous copy of the gene had been deleted was isolated (data not shown). Dominant-activating (G45R) and dominant-interfering (G207R) *gasC* alleles were created by inverse PCR (see MATERIALS AND METHODS). The glycine 45 to arginine mutation is predicted to inactivate the GTPase activity of GasC, resulting in a GTP-bound Gα-subunit that constitutively signals. In contrast, the glycine 207 to arginine mutation is predicted to block the conformational switch that accompanies GTP binding and is necessary for Gβγ release, resulting in the Gα-subunit being unable to signal (KAZIRO *et al.* 1991; KURJAN *et al.* 1991). The plasmids carrying the dominant-activating $gasC^{G45R}$ allele (pSZ5135), the dominant-

TABLE 1

Fungal strains used in this study

Species	Strain	Genotype	Plasmid copy number	Reference
P. marneffei	FRR2161	Wild type		American Type Culture Collection strain
P. marneffei	SPM4	*niaD1; pyrG1*		Borneman *et al.* (2001)
P. marneffei	TS32-7-8	*niaD1; pyrG1;* Δ*gasC::pyrG*		This study
P. marneffei	TS43-5-12	*niaD1; pyrG1; gasC^{G207R} pyrG* [pSZ5136]	~2	This study
P. marneffei	TS32-7-4	*niaD1; pyrG1; gasC^{G207R} pyrG* [pSZ5136]	~10	This study
P. marneffei	TS43-5-2	*niaD1; pyrG1; gasC^{G207R} pyrG* [pSZ5136]	~8	This study
P. marneffei	TS32-5-4	*niaD1; pyrG1; gasC^{G45R} pyrG* [pSZ5135]	~3	This study
P. marneffei	TS32-5-15	*niaD1; pyrG1; gasC^{G45R} pyrG* [pSZ5135]	~15	This study
P. marneffei	TS32-4-6	*niaD1; pyrG1; gasC^{G45R} pyrG* [pSZ5135]	~10	This study
P. marneffei	TS32-3-4	*niaD1; pyrG1; gasC pyrG* [pSZ5134]	~8	This study
P. marneffei	TS51-4-23	*niaD1; pyrG1; gasC^{G207R} gasA^{G203R} pyrG* [pSZ5134]	~5	This study
P. marneffei	TS51-3-8	*niaD1; pyrG1; gasC^{G45R} gasA^{G42R} pyrG* [pSZ5134]	~5	This study
A. nidulans	MH1	*biA1*		Glasgow wild type
A. nidulans	A770	*pyrG89; pabaB22; riboB2*		Fungal Genetics Stock Center
A. nidulans	TSA770.N3	*pyrG89; pabaB22; riboB2; gasC^{G207R} pyrG* [pSZ5136]	~3	This study
A. nidulans	TSA770.D19	*pyrG89; pabaB22; riboB2; gasC^{G45R} pyrG* [pSZ5135]	~10	This study
A. nidulans	TSA770.D23	*pyrG89; pabaB22; riboB2; gasC^{G45R} pyrG* [pSZ5135]	~2	This study
A. nidulans	TSA770.W3	*pyrG89; pabaB22; riboB2; gasC pyrG* [pSZ5134]	~10	This study

Brackets indicate the plasmid carrying the preceding allele, which has been introduced by transformation.

interfering *gasC^{G207R}* allele (pSZ5136), and the wild-type *gasC* allele (pSZ5134) were transformed into the strain SPM4 and transformants were isolated by direct selection for *pyrG*$^+$. Southern blot analysis on genomic DNA from these transformants was performed to estimate the number of integrated plasmid copies present in each transformant, as indicated in Table 1. Both low- and high-copy-number transformants were analyzed. Attempts to introduce the wild-type and dominant mutant alleles of *gasC* into the null background did not yield any transformants and further experimentation showed that protoplasts of this strain were severely compromised in their capacity to regenerate (data not shown).

GasC-mediated signaling affects conidiation: To determine the effect of GasC on conidiation, the colony morphology of the different *gasC* mutant strains was examined (Figure 3A). In the Δ*gasC* mutant, conidiation was denser and more uniform across the colony compared to the wild type, while strains carrying the dominant-activating *gasC^{G45R}* allele were delayed in conidiation and produced excessive aerial hyphae, resulting in a clustered and nonuniform conidiation pattern. These phenotypes were more severe in transformants carrying high numbers of the dominant-activating *gasC^{G45R}* allele. In strains carrying high numbers of the wild-type *gasC* allele, the effects on conidiation were similar to those observed using the dominant-activating *gasC^{G45R}* allele, although less severe (data not shown). The conidiation pattern of the strains carrying the dominant-interfering *gasC^{G207R}* allele was very similar to wild-type *P. marneffei*, irrespective of plasmid copy number.

To quantitate the effect of GasC on conidiation, conidial yields of each of the *gasC* mutant strains were examined under various conditions including hyperosmotic conditions and different nitrogen sources. Hyperosmotic growth conditions (0.3 M NaCl) lowered the conidial yield of the wild type, the Δ*gasC* mutant, and strains expressing the dominant-interfering *gasC^{G207R}* allele compared to the effects of normal osmotic conditions, but no significant differences in conidial yields between these strains were evident (Figure 3B). In contrast to many other fungal species, *P. marneffei* is sensitive to higher concentrations of salt and cannot grow on 1 M NaCl (S. Zuber and A. Andrianopoulos, unpublished data). Under normal osmotic conditions, the strains carrying the dominant-activating *gasC^{G45R}* allele produced approximately five times fewer conidia than the other strains produced, consistent with its clustered and nonuniform conidiation pattern (see above), while hyperosmotic conditions (0.3 M NaCl) resulted in a complete lack of conidiation (Figure 3B). Very similar responses were also observed on 0.3 M KCl and 0.5 M sorbitol (data not shown). Furthermore, in the presence of 0.3 M NaCl the onset of conidiation was initiated earlier in the Δ*gasC* mutant than in the wild type. Dense conidiation was visible after 4 days in the Δ*gasC* mutant, whereas no conidiophores were present in the wild type at this point in time (data not shown). In *P. marneffei*, conidiation varies depending on the nitrogen source. Conidiation is greater when grown on medium containing GABA as a sole nitrogen source than when grown on ammonium (A. Borneman and A. Andriano-

POULOS, unpublished data). Quantitation of conidial yields showed that all strains produced roughly five times fewer conidia on ammonium than on GABA (Figure 3C), suggesting that the regulation of conidiation by GasC is in a pathway independent of nitrogen source sensing.

Conidiation in *P. marneffei* is also influenced by carbon limitation. Under carbon-limiting conditions (0.1% glucose), conidiophores are precociously formed within 2 days, whereas under carbon-sufficient conditions (1% glucose) conidiophores appear only after 5 days (ANDRIANOPOULOS 2002). The Δ*gasC* mutant and strains carrying the dominant-interfering $gasC^{G207R}$ allele displayed the same conidiation characteristics as the wild type displayed on low and high glucose after 3 days incubation. In contrast, strains carrying the dominant-activating $gasC^{G45R}$ allele failed to produce conidiophores after 3 days on either low or high glucose. This is consistent with the delay in conidiation of these strains (see above) and shows that the expression of the dominant-activating $gasC^{G45R}$ allele leads to a delay in conidiation irrespective of the carbon status (data not shown).

GasC is involved in the regulation of secondary metabolite production: *P. marneffei* produces a red pigment during filamentous growth at 25°. Nutritional conditions, especially the nitrogen source, have a significant impact on the amount of this secondary metabolite produced (ANDRIANOPOULOS 2002). On medium containing GABA as a sole nitrogen source, the Δ*gasC* mutant and strains carrying the dominant-interfering $gasC^{G207R}$ allele produced less pigment than the wild type produced and appeared white. In contrast, strains carrying the dominant-activating $gasC^{G45R}$ allele showed an overproduction of red pigment relative to the wild type and appeared dark red (Figure 4). All strains produced more pigment on medium containing glutamine as a sole nitrogen source than on GABA, but the differences between the strains were similar to those on GABA (data not shown). When ammonium was the sole nitrogen source, all strains failed to produce red pigment such that the wild-type, Δ*gasC* mutant, and dominant-interfering $gasC^{G207R}$ mutant strains appeared white while the

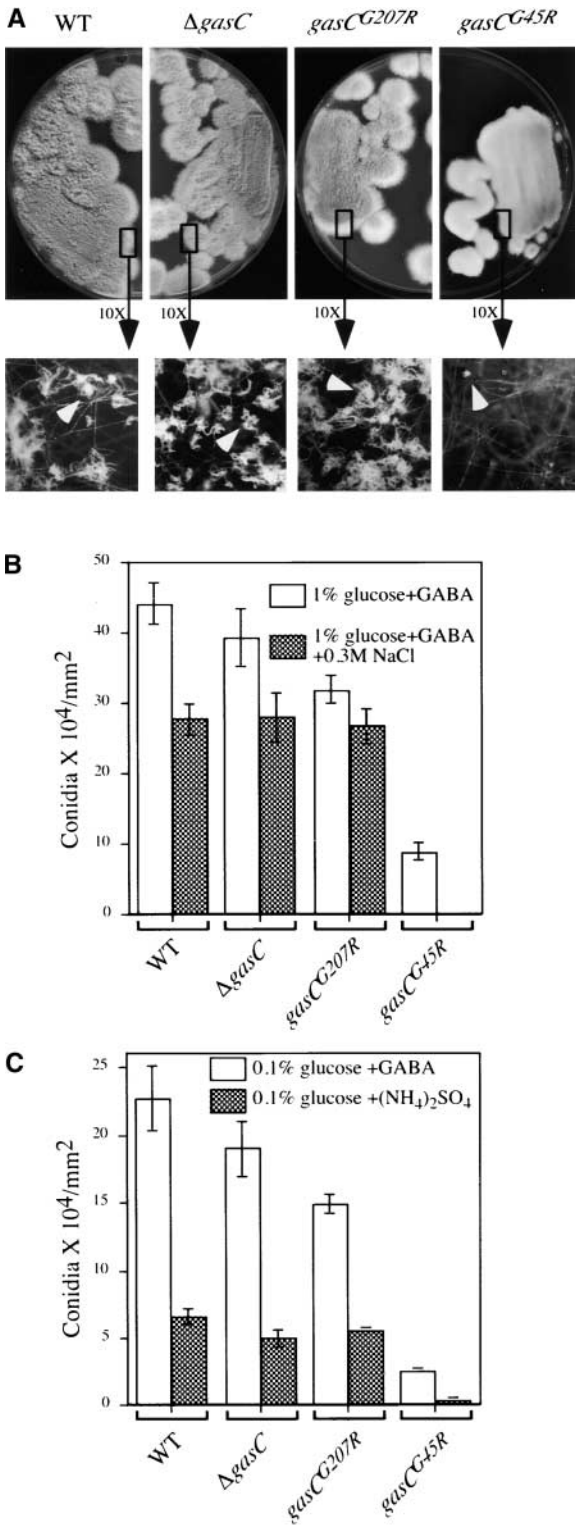

FIGURE 3.—GasC affects conidiation. (A) Colonial morphology of *P. marneffei* wild type, Δ*gasC* mutant, dominant-interfering $gasC^{G207R}$ (TS32-7-4), and dominant-activating $gasC^{G45R}$ (TS32-5-1) strains. The strains were grown on ANM + GABA for 10 days at 25°. Plates showing colony morphology are shown (above) and magnified sections (×10) depict conidiophores (below). White arrowheads indicate conidiophore structures. (B and C) Quantitation of conidial yield in the wild-type, Δ*gasC* mutant, dominant-interfering $gasC^{G207R}$, and dominant-activating $gasC^{G45R}$ strains under hyperosmotic conditions (B) and different nitrogen sources (C). Conidia (1×10^5) of each strain were spread on 1% ANM + GABA plates (open bars in B), 1% ANM + GABA + 0.3 M NaCl plates (cross-hatched bars in B), 0.1% ANM + GABA plates (open bars in C), and 0.1% ANM + $(NH_4)_2SO_4$ plates (cross-hatched bars in C) and incubated at 25° for 8 days. Subsequently, conidia from a defined area (2.5 cm^2) were harvested for counting. Values are the average of three replicates and standard errors are indicated. Values indicated for the dominant-interfering $gasC^{G207R}$ (TS32-7-4, TS43-5-2) and the dominant-activating $gasC^{G45R}$ (TS32-5-1, TS32-4-6) strains are an average of two independent transformants.

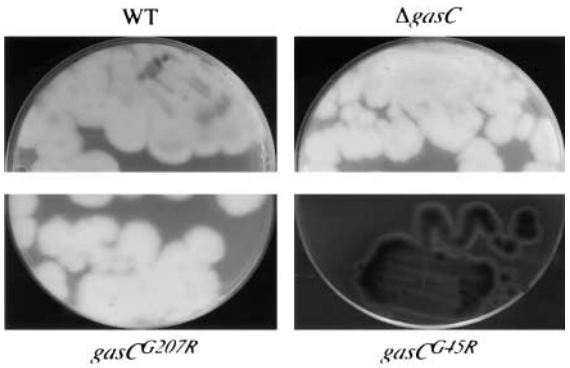

Figure 4.—GasC regulates the production of red pigment. *P. marneffei* wild type, Δ*gasC* mutant, dominant-interfering *gasC*^{G207R} (TS32-7-4), and dominant-activating *gasC*^{G45R} (TS32-5-1) strains were grown on 1% ANM + GABA for 10 days at 25°. Images were captured from the underside of the plate. Colonies appear dark when red pigment is present.

strains carrying the dominant-activating *gasC*^{G45R} allele showed a slight yellow coloration (data not shown).

GasC plays a major role in conidial germination: After 10 hr of incubation at 25°, 25% of wild-type conidia have germinated and show a germ tube (Figure 5A). Strains carrying the dominant-activating *gasC*^{G45R} allele showed an accelerated germination rate compared to that of the wild type, such that 20% of the conidia had produced a germ tube after 6 hr incubation, whereas strains expressing the dominant-interfering *gasC*^{G207R} allele showed a slower germination rate compared to that of the wild type. The Δ*gasC* mutant was severely delayed in germination, requiring up to 27 hr to achieve 25% germination. In contrast to the wild type and the other strains that exhibit relatively synchronous conidial germination, the conidia of the Δ*gasC* mutant germinated asynchronously with some conidia failing to produce a germ tube after 40 hr (Figure 5B). The severity of the germination defect for strains carrying the dominant-activating *gasC*^{G45R} allele was copy-number dependent, such that high-copy-number transformants germinated faster than low-copy-number transformants. High-copy-number transformants carrying the dominant-interfering *gasC*^{G207R} allele germinated as quickly as the wild type and only low-copy-number transformants showed a delay in germination compared to the wild type (data not shown).

To investigate the role of GasC in germination further, we followed germination kinetics of the different *gasC* mutant strains under various nutritional conditions. The germination kinetics of the wild type and the *gasC* mutants under carbon-limiting (0.1% glucose) *vs.* carbon-sufficient conditions (1% glucose) were identical (data not shown). When ethanol was compared to glucose as sole carbon source, all strains germinated more slowly on ethanol and the kinetic shift in the germination rate on glucose *vs.* ethanol was very similar for all strains (Figure 5C). If GasC plays a role in sensing the presence of a carbon source, strains carrying the dominant-activating *gasC*^{G45R} allele should be able to germinate in the absence of any carbon source. However, even after 48 hr, none of the strains showed any signs of germination. The conidia did not appear swollen and did not show a germ tube (data not shown).

In *A. nidulans*, cAMP signaling has recently been shown to be involved in spore germination (Fillinger *et al.* 2002). To test if elevated levels of cAMP could suppress the delay in germination observed in the Δ*gasC* mutant, dbcAMP was used in conjunction with the phosphodiesterase inhibitor theophylline and germination of the *gasC* mutant strains was examined. The kinetics of spore germination for any given strain were identical in the presence or absence of dbcAMP and theophylline (data not shown), suggesting that GasC in *P. marneffei* may not signal through the cAMP-PKA pathway. Alternatively, the compounds may not be able to efficiently enter ungerminated spores.

GasC is not required for yeast growth at 37°: The morphology of yeast colonies for the Δ*gasC* mutant and strains carrying the dominant-interfering *gasC*^{G207R} allele were indistinguishable from wild-type yeast colonies. However, strains carrying the dominant-activating *gasC*^{G45R} allele exhibited an increase in filamentation and invasive growth at the colony edges compared to the wild type (Figure 6A). In addition, the Δ*gasC* mutant produced slightly smaller colonies than those the wild type produced. This probably reflects the fact that spores of the Δ*gasC* mutant germinate less efficiently, leading to some smaller yeast colonies. Prolonged incubation allowed these colonies to reach wild-type size, supporting the hypothesis that this phenotype is due to the germination defect and not to an additional growth defect. Furthermore, no differences in growth were observed between the different *gasC* mutants in hyperosmotic conditions (0.3 M NaCl) at 37° (data not shown).

The different *gasC* mutant strains were incubated at 37° for 4 days and moved to 25° to induce the yeast-hyphal dimorphic switch. Like the wild type, all strains produced a filamentous colony periphery after 24 hr, showing that GasC is not required for the yeast-to-hyphal dimorphic switch. Similarly, the hyphal-to-yeast dimorphic switch was investigated by examining the production of yeast cells at 37° microscopically. All the *gasC* mutants were able to produce yeast cells that were indistinguishable from the yeast cells produced by the wild type, indicating that this developmental pathway is not affected in any of the *gasC* mutant strains (Figure 6B). Together, these results indicate no major role for GasC in the dimorphic switch or the maintenance of yeast growth at 37°.

Characterization of *P. marneffei* strains carrying *gasA* and *gasC* mutations: We have previously shown that the Gα-subunit GasA in *P. marneffei* is a key regulator of conidiation and to a lesser extent also regulates the production of red pigment produced by *P. marneffei* at 25° (Zuber *et al.* 2002). Both of these processes are

also regulated by the Gα-subunit GasC (see above). To examine the relationship between these two Gα-subunits, double dominant-activating [*gasA*^{G42R}, *gasC*^{G45R}] and double dominant-interfering [*gasA*^{G203R}, *gasC*^{G207R}] mutants were produced using a plasmid carrying both dominant-activating *gasA*^{G42R} and *gasC*^{G45R} alleles (pSZ5447) and a plasmid carrying both dominant-interfering *gasA*^{G203R} and *gasC*^{G207R} alleles (pSZ5448; see MATERIALS AND METHODS). These plasmids were transformed into *P. marneffei* and transformants isolated by direct selection for *pyrG*$^{+}$.

At 25° the double dominant-activating [*gasA*^{G42R}, *gasC*^{G45R}] mutants displayed thick aerial hyphae and completely lacked conidiophores and conidia, as observed in the dominant-activating *gasA*^{G42R} mutants alone (ZUBER *et al.* 2002). These strains also showed overproduction of the red pigment as noted for the dominant-activating *gasC*^{G45R} mutant alone (see above). However, they did not show any phenotype additional to that observed in either of the single mutants (data not shown). The double dominant-interfering [*gasA*^{G203R}, *gasC*^{G207R}] mutants were impaired in the production of the red pigment and appeared white, but otherwise similar to the wild type (data not shown). At 37° the morphology of yeast colonies for the double dominant-interfering [*gasA*^{G203R}, *gasC*^{G207R}] mutants was indistinguishable from wild-type yeast colonies. The double dominant-activating [*gasA*^{G42R}, *gasC*^{G45R}] mutants, however, exhibited an increase in filamentation and invasive growth at the colony edges, as observed in the dominant-activating *gasC*^{G45R} mutants alone (see above). Together, this shows that the double mutants exhibited no observable additive effects.

GasC function is conserved in *A. nidulans*: The high homology between GasC and the uncharacterized *A. nidulans* homolog GanB suggests that the two proteins may play a similar role (Figure 1B). To assess the role of GasC in *A. nidulans*, the plasmids carrying the dominant-

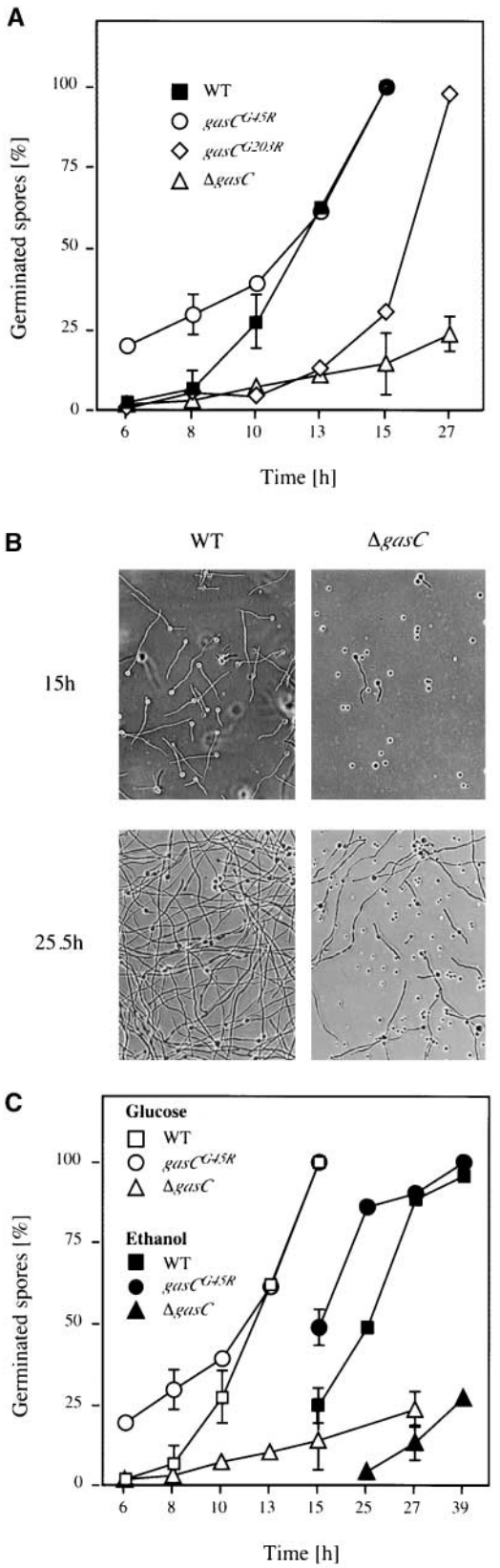

FIGURE 5.—GasC plays a major role in conidial germination. (A) Kinetics of conidial germ tube outgrowth in *P. marneffei* wild type, Δ*gasC* mutant, dominant-interfering *gasC*^{G207R} (TS43-5-12), and dominant-activating *gasC*^{G45R} (TS32-5-4) strains. Conidia (2×10^6) of each strain were inoculated into 300 μl 0.1% ANM + $(NH_4)_2SO_4$ and incubated at 25°. Conidia showing germ tube outgrowth were counted in at least two independent microscopic fields at the time points specified. Results are expressed as percentage of conidia per field. Results are representative of three different experiments and standard deviations are indicated. (B) Asynchronous germination in the *P. marneffei* Δ*gasC* mutant compared to wild type. Conidia (2×10^6) of each strain were inoculated into 300 μl 0.1% ANM + $(NH_4)_2SO_4$. Representative pictures obtained by light microscopy after 15 and 25.5 hr incubation at 25° are shown. (C) Kinetics of germ tube outgrowth in *P. marneffei* wild type, Δ*gasC* mutant, and dominant-activating *gasC*^{G45R} (TS32-5-4) on ethanol compared to glucose. Conidia (2×10^6) of each strain were inoculated into 300 μl CF + EtOH + $(NH_4)_2SO_4$ (solid symbols) and 0.1% ANM + $(NH_4)_2SO_4$ (open symbols), respectively, and incubated at 25°. Conidia showing a germ tube outgrowth were counted in at least two independent microscopic fields at the time points specified. Results are expressed as the percentage of conidia per field and are representative of two different experiments. Standard deviations are indicated.

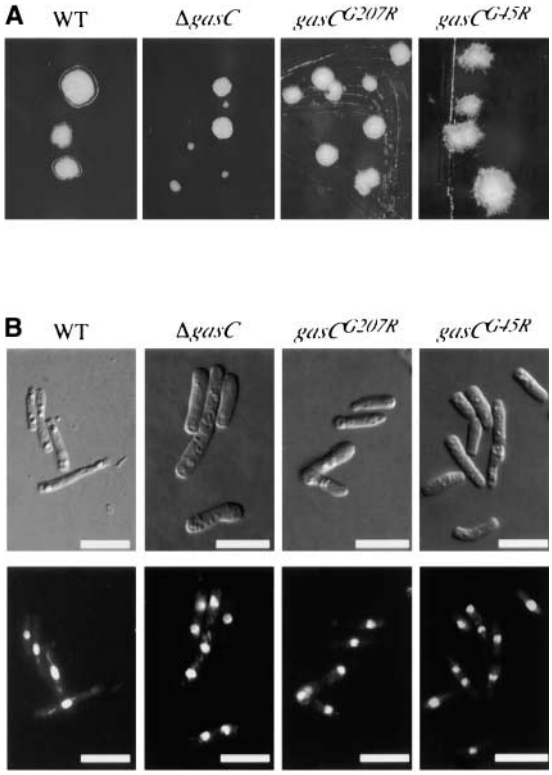

FIGURE 6.—GasC is not required for yeast growth at 37°. Colonial morphologies of *P. marneffei* wild-type, Δ*gasC* mutant, dominant-interfering *gasC*^{G207R} (TS43-5-12), and dominant-activating *gasC*^{G45R} (TS32-5-4) strains. The strains were grown on SD at 37° for 4 days. The colony edges of the dominant-activating *gasC*^{G45R} strain are slightly more filamentous compared to those of the wild type. (B) Microscopic examination of *P. marneffei* wild type, Δ*gasC* mutant, dominant-interfering *gasC*^{G207R} (TS43-5-12), and dominant-activating *gasC*^{G45R} (TS32-5-4) strains. Strains were grown on BHI at 37° for 4 days. Bars, 20 μm. (Top) DIC and (bottom) DAPI-stained epifluorescence of nuclei.

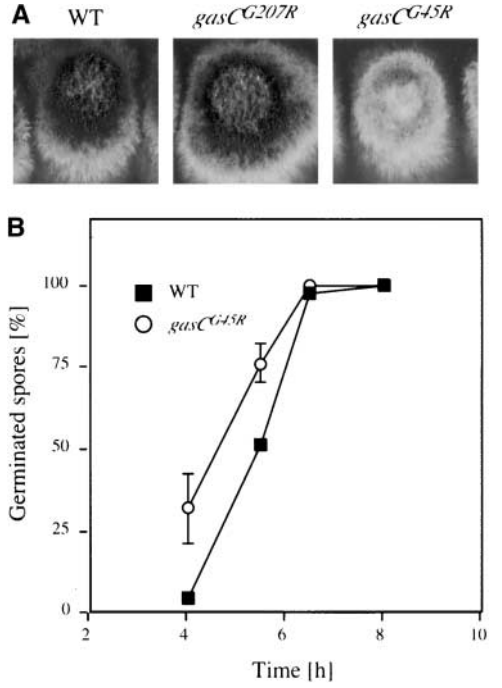

FIGURE 7.—GasC function is conserved in *A. nidulans*. (A) Colonial morphology of *A. nidulans* wild type, a transformant carrying the *P. marneffei* dominant-interfering *gasC*^{G207R} allele (TSA770.N3), and a transformant carrying the *P. marneffei* dominant-activating *gasC*^{G45R} allele (TSA770.D23). The level of conidiation is reflected by darker coloration of the colony. Strains were grown on 1% ANM + NH_4T for 2 days at 37°. (B) Kinetics of germ tube outgrowth in *A. nidulans* wild type and transformants carrying the *P. marneffei* dominant-activating *gasC*^{G45R} allele. Conidia (2×10^6) of each strain were inoculated into 300 μl of 0.1% ANM + $(NH_4)_2SO_4$ at 37° and conidia showing a germ tube were counted in at least two independent microscopic fields at the time points specified. Results are expressed as a percentage of conidia per field. Results are representative of two different experiments and standard deviations are indicated. Values indicated for the *gasC*^{G45R} strains are an average of two independent transformants (TSA770.D23, TSA770.D19).

activating *gasC*^{G45R} allele (pSZ5135) and the dominant-interfering *gasC*^{G207R} allele (pSZ5136) were transformed into the *A. nidulans* strain A770 and transformants were isolated by direct selection for *pyrG*$^+$. Southern blot analysis of genomic DNA for these transformants was performed to estimate the number of plasmid copies integrated in each transformant (data not shown).

The *A. nidulans* wild type and strains carrying the dominant-interfering *gasC*^{G207R} allele were indistinguishable from each other. Strains carrying the dominant-activating *gasC*^{G45R} allele, however, showed a delay in conidiation and a reduced number of conidia when compared to the wild type (Figure 7A). Similar to *P. marneffei*, this phenotype was copy-number dependent and easily detectable only in high-copy-number transformants (data not shown). Monitoring the kinetics of germ tube outgrowth in *A. nidulans* wild type showed that after 6 hr of incubation at 37° 50% of conidia had begun to germinate and showed a germ tube (Figure 7B). The strains carrying the *P. marneffei* dominant-activating *gasC*^{G45R} allele showed an accelerated germination rate, such that 30% of the conidia had produced a germ tube after only 4 hr incubation. Similar to *P. marneffei*, the severity of the germination defect for strains carrying the dominant-activating *gasC*^{G45R} allele was copy-number dependent, such that high-copy-number transformants germinated faster than low-copy-number transformants. Taken together, these results show that GasC plays the same role in *A. nidulans* as it does in *P. marneffei* and suggest that GanB may play a role in *A. nidulans* similar to the one GasC plays in *P. marneffei*.

DISCUSSION

GasC is a class III fungal Gα-subunit involved in germination: We have identified a new G-protein α-subunit encoding gene, *gasC*, in the dimorphic fungus *P. marneffei*. GasC is a class III fungal Gα-subunit similar to *S. cerevisiae*

Gpa2, *S. pombe* Gpa2, *Ustilago maydis* Gpa3, *Cryptococcus neoformans* Gpa1, *Neurospora crassa* Gna3, and others (Nakafuku *et al.* 1988; Isshiki *et al.* 1992; Tolkacheva *et al.* 1994; Regenfelder *et al.* 1997; Bölker 1998; Kays *et al.* 2000). We have shown that GasC plays a major role in conidial germination in *P. marneffei.* This is the first report showing that a Gα-subunit is involved in germination in filamentous fungi. A Δ*gasC* mutant is severely delayed in germination, whereas strains carrying a dominant-activating $gasC^{G45R}$ allele, leading to constitutive activation of the signaling pathway, show a significantly accelerated germination rate compared to that of the wild type. The involvement of a Gα-subunit in germination is of considerable interest due to its potential involvement in sensing and transmitting the signal to germinate, a hitherto poorly understood process.

GasC does not mediate carbon source sensing during germination: The presence of water and a fermentable carbon source such as glucose is sufficient to activate a spore and to trigger spore germination in many fungi (d'Enfert 1997; Herman and Rine 1997; Osherov and May 2001). In *S. pombe* the GasC homolog Gpa2 mediates glucose sensing and is crucial for ascospore germination (Hatanaka and Shimoda 2001). In contrast, the results presented here suggest that GasC plays either a minor role or no role in transmitting a carbon source signal during germination. This conclusion is based on the observation that strains carrying the dominant-activating $gasC^{G45R}$ allele are not able to germinate in the absence of any carbon source. In support of this is the fact that all strains germinated more slowly on ethanol than on glucose (Figure 5C), suggesting that if GasC was the main glucose sensor, strains carrying the dominant-activating $gasC^{G45R}$ allele would be expected to show the same germination rate on any carbon source by bypassing the requirement for the activating signal. This result suggests a fundamental difference between yeasts and filamentous fungi in the signaling components used for glucose sensing. The observation that the Δ*gasC* mutant spores are not completely blocked in germination and will eventually germinate is a strong indication that GasC is linked to a very early sensor triggering germination and that downstream factors or alternate pathways can compensate for the absence of GasC signaling. A likely alternate pathway is that involving Ras. *A. nidulans* strains overproducing a dominant-activating *rasA* allele produce giant swollen spores with multiple nuclei that fail to produce germ tubes, demonstrating the critical role of RasA in the later events of spore germination such as progression from isotropic to polarized growth (Som and Kolaparthi 1994). Whether RasA also plays a role during initiation of germination is unclear at this stage (Osherov and May 2000; Fillinger *et al.* 2002). Therefore, we propose that GasC signaling is crucial for activation of the spore and that other signaling pathways such as the Ras pathway are required for postinitiation

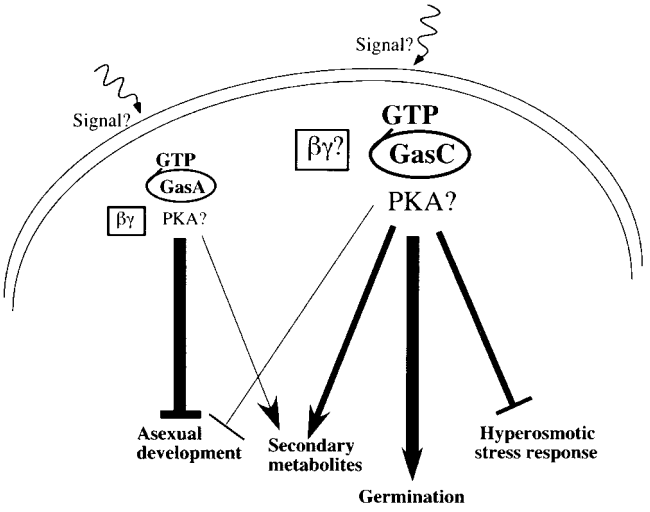

Figure 8.—Proposed model for signal transduction pathways regulating germination, conidiation, and secondary metabolite production in *P. marneffei.* In *P. marneffei*, the Gα-subunit GasC plays a major role in the regulation of germination. GasC also regulates secondary metabolites and to a lesser extent conidiation, as well as being involved in the response to hyperosmotic conditions. The Gα-subunit GasA is a key regulator of conidiation and to a lesser extent also regulates the production of the red pigment (Zuber *et al.* 2002). The degree of regulation of a certain process by GasC and GasA is indicated by the thickness of the line.

events of the germination process. This model predicts that strains carrying the dominant-activating $gasC^{G45R}$ allele should initiate germination even in the absence of a carbon source. One possible explanation why this was not evident is if the dominant-activating $gasC^{G45R}$ strains initiate germination, but in the absence of a carbon source never reach the isotropic or polarized growth stages. Early markers of germination would be needed to further dissect the role of GasC in the germination process (Fillinger *et al.* 2002; Osherov *et al.* 2002).

GasC-mediated signaling affects secondary metabolite production and conidiation: The Gα-subunit GasC not only is involved in germination, but also plays a role in the regulation of secondary metabolite production and conidiation (Figure 8). Dominant-activating $gasC^{G45R}$ strains positively affect the production of the red pigment by *P. marneffei* at 25° (Figure 4). Furthermore, the effects of the dominant-activating $gasC^{G45R}$ allele suggest that GasC signaling negatively affects the onset of conidiation and the conidial yield (Figure 3). In *S. cerevisiae* Gpa2 is linked to a glucose sensing receptor and is involved in the transmission of the signal for nitrogen starvation (Kübler *et al.* 1997; Lorenz and Heitman 1997; Colombo *et al.* 1998; Kraakman *et al.* 1999). In contrast to the situation in *S. cerevisiae*, GasC does not appear to be involved in sensing the quality of the nitrogen source present in the medium, as all strains were able to respond to changes in nitrogen source and to alter the production of red pigment. Similarly, the fact

that the conidial yield of all strains was lowered by the same factor on ammonium *vs.* GABA indicates that the regulation of conidiation by GasC is independent of the nitrogen source (Figure 3C). GasC does not appear to be linked to a sensor for carbon limitation in the initiation process of conidiation. In wild-type *P. marneffei* the formation of conidiophores is delayed by high levels of glucose. The Δ*gasC* mutant would have been expected to precociously conidiate irrespective of the carbon status if GasC signaling was inhibiting conidiation by responding to conditions of carbon sufficiency.

This suggests that conidiation may be initiated by a more general stress response rather than by a specific response to carbon. In this respect, the phenotypes exhibited by the *gasC* mutants under hyperosmotic conditions are of particular interest. Strains carrying the dominant-activating $gasC^{G45R}$ allele are completely blocked in conidiation under these conditions (Figure 3B) and the onset of conidiation in the Δ*gasC* mutant is early compared to the wild type. Together these effects suggest an active role for GasC in transmitting the signal leading to hyperosmotic stress response and suggests that class III fungal Gα-subunits not only are involved in transmitting nutritional signals as in *S. cerevisiae* and *S. pombe*, but also might be involved in transmitting a wider range of signals depending on the environment encountered by a given species. In the corn smut fungus *U. maydis*, the Gα-subunit Gpa3, a homolog of *S. cerevisiae* Gpa2, regulates mating, dimorphic switching, and pathogenicity. In *U. maydis*, a Δ*gpa3* mutant cannot respond to a pheromone, whereas a constitutively active *gpa3* allele allows pheromone-independent mating, demonstrating that Gpa3 plays an active role in transmitting the pheromone signal. Furthermore, the Δ*gpa3* mutant is nonpathogenic and Gpa3 has been proposed to transmit signals coming from the host plant (Regenfelder *et al.* 1997; Krüger *et al.* 1998). Similarly, in the human pathogen *C. neoformans*, another class III fungal Gα-subunit, Gpa1, regulates processes such as mating and virulence. The *C. neoformans* Gpa1 has been proposed to be linked to sensors of glucose, nitrogen, and iron deprivation. The specific receptors that initiate these signaling cascades and their link to Gpa1, however, remain to be elucidated (Tolkacheva *et al.* 1994; Alspaugh *et al.* 1997). Our data also indicate no major role for GasC in the dimorphic switch or the maintenance of yeast growth at 37° (Figure 6). This is in contrast to the situation in *S. cerevisiae* and *U. maydis* where Gpa2 and Gpa3 are required for pseudohyphal growth and dimorphic switching, respectively (Kübler *et al.* 1997; Lorenz and Heitman 1997; Regenfelder *et al.* 1997) and further emphasizes the diversity of processes regulated by the class III fungal Gα-subunits.

GasC signaling and the cAMP-PKA pathway: The signal transduction pathway downstream of GasC remains to be determined. Of particular interest, however, is the fact that the Δ*gasC* mutant shows a delay in germination and a highly asynchronous germination pattern that resembles the delay and asynchronous germination pattern seen in the *A. nidulans* Δ*cyaA* mutant (Figure 5, A and B; Fillinger *et al.* 2002). A delay in germination is also seen in the *A. nidulans* Δ*pkaA* mutant (Fillinger *et al.* 2002). In addition to germination, PkaA in *A. nidulans* also plays a role in the production of secondary metabolites and conidiation (Shimizu and Keller 2001), which are the three processes regulated by GasC in *P. marneffei*. This supports a model in which GasC signals through the cAMP-PKA pathway (Figure 8). The observation that dbcAMP and theophylline were unable to suppress the delay in the germination rate of the Δ*gasC* mutant may be due to the impermeability of ungerminated spores, as has been suggested for similar studies (Osherov and May 2000).

Overlapping roles of the two Gα-subunits GasA and GasC: In *P. marneffei* GasA-mediated signaling blocks conidiation and to a lesser extent regulates production of the red pigment (Zuber *et al.* 2002). Conversely, GasC is a weaker regulator of conidiation, but a stronger regulator of pigment production. The overlapping roles between these two Gα-subunits may suggest that they share downstream signaling components or effectors. The weak regulation of conidiation through GasC and pigment production through GasA could be indirect effects resulting from cross-activation of the main programs regulated by each of the Gα-subunits. In support of this are the phenotypes displayed by the strains carrying the dominant-interfering $gasC^{G207R}$ allele, which suggest that this mutation blocks signaling only partially. High-copy-number transformants show a slightly but significantly lower yield in conidiation compared to the wild type and Δ*gasC* mutant (Figure 3, B and C), while low-copy-number transformants are not as delayed in germination as the Δ*gasC* mutant (Figure 5A). Overexpression of the dominant-interfering $gasC^{G207R}$ allele may result in an interaction with the βγ-subunit that normally interacts with GasA leading to enhanced GasA signaling, which would explain the lower conidial yield observed in the dominant-interfering $gasC^{G207R}$ strains. Alternatively, it has recently been shown that *S. cerevisiae* Gpa2 interacts with a member of a new class of β-subunits composed of kelch repeats, which are predicted to fold into structures strikingly similar to the WD-40-based β-subunits (Harashima and Heitman 2002). If GasC also interacts with this new class of β-subunit, the interaction could be different at the molecular level, making the dominant-interfering $gasC^{G207R}$ mutation less efficient than that in a Gα-subunit that associates with a classic Gβ-subunit. Overlapping roles for Gα-subunits have previously been noted in *N. crassa* where conidiation is regulated by Gna1 and Gna3 belonging to class I and III fungal Gα-subunits, respectively. Similar to the Δ*gasC* mutant, the Δ*gna3* mutant exhibits shorter aerial hyphae and premature dense conidiation. The Δ*gna1*

mutant also shows shorter aerial hyphae and is delayed in conidiation (Ivey *et al.* 1996; Kays *et al.* 2000).

The Gα-subunit FadA from *A. nidulans* is a close homolog of GasA and is known to regulate conidiation and secondary metabolism (Yu *et al.* 1996; Hicks *et al.* 1997). *A. nidulans* FadA has been shown to signal both through PkaA and in a PkaA-independent fashion (Shimizu and Keller 2001). On the basis of our observation that GasC functions similarly in both *A. nidulans* and *P. marneffei* (Figure 7), we speculate that GanB might play a role in *A. nidulans* similar to the one GasC plays in *P. marneffei* and that both might have a role in cAMP-PKA signaling. This makes the relationship between GasA, GasC, and the signal transduction pathways transmitting their respective signals very intriguing. The characterization of the *gasA, gasC* double mutant did not detect any synergistic effects, suggesting that GasA and GasC act independently and probably respond to different environmental cues to coordinately regulate conidiation and pigment production (Figure 8). To identify the specific signals and receptors linked to both GasA and GasC, as well as the signaling pathways used to transmit these signals to downstream effectors, is an important issue.

This work was supported by a grant from the Australian Research Council and Novozyme A/S. S. Zuber was supported by an International Postgraduate Research Scholarship and a Melbourne Research Scholarship.

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Communicating editor: M. S. Sachs