Applied and Environmental Microbiology, July 2011, p. 4700–4703 Vol. 77, No. 13
0099-2240/11/$12.00 doi:10.1128/AEM.00243-11

Phylogenetic and Functional Analysis of *Aspergillus fumigatus* MGTC, a Fungal Protein Homologous to a Bacterial Virulence Factor▽†

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Received 3 February 2011/Accepted 8 May 2011

MgtC is important for the survival of several bacterial pathogens in macrophages and for growth under magnesium limitation. Among eukaryotes, a gene homologous to *mgtC* was found only in the pathogenic fungus *Aspergillus fumigatus*. Our data show that the *A. fumigatus* MgtC (AfuMgtC) protein does not have the same function as the bacterial MgtC proteins.

Although *Aspergillus fumigatus* has become the most common and dangerous airborne fungal pathogen of humans, the molecular determinants responsible for its virulence remain largely unknown (19). A current hypothesis is that in *A. fumigatus*, specific genes would code for essential virulence factors for this fungal species. One of these genes, AFUA_7G05060, was of major interest since it was homologous to an essential bacterial virulence factor, MgtC (for magnesium transporter C).

MgtCp has been shown to be a virulence factor important for the proliferation of several intracellular bacterial pathogens in macrophages (1, 4, 27). The function of MgtCp is unknown, but it seems to facilitate or regulate Mg^{2+} transport since it is required for growth at low Mg^{2+} concentrations (11). MgtC-like proteins are found in a limited number of bacterial genomes, and phylogenetic analysis suggests that MgtC has been acquired by horizontal gene transfer (HGT) repeatedly throughout bacterial evolution.

Like bacterial pathogens, conidia of *A. fumigatus* are also phagocytosed by macrophages and they have to germinate intracellularly to establish disease. In addition, divalent cations such as Zn^{2+} and Fe^{3+} are required for *A. fumigatus* growth (30, 31). Magnesium is the most abundant divalent cation in cells and is involved in many cellular functions as a cofactor in numerous enzymatic reactions, as well as being necessary for the stability of plasma membranes. Until now, the growth of *A. fumigatus* under magnesium limitation has not been investigated and magnesium transporters have not been identified.

Genomic analysis of the *mgtC* homologs in *A. fumigatus*. BLAST analysis (http://blast.ncbi.nlm.nih.gov/) identified a unique MgtC-like protein in *A. fumigatus*. *A. fumigatus MGTC* (Afu*MGTC*) is 840 bp long and encodes a 280-amino-acid protein with a theoretical molecular mass of 31 kDa. MGTCp has four predicted transmembrane helices and one putative N-glycosylation site. The amino acid sequence is 33% identical to the protein of *Salmonella enterica* serovar Typhimurium and 30% identical to the protein of *Mycobacterium tuberculosis*. Like its bacterial orthologs (5), the MgtC protein of *A. fumigatus* contains the conserved hydrophobic "MgtC domain" located in the N-terminal part of the protein (Fig. 1) whereas the C-terminal region of the *A. fumigatus* protein is not conserved.

Interestingly, this homolog of bacterial MgtC is unique in the fungal kingdom since no *MGTC* homolog was identified by a BLAST search in other eukaryotic species or in *Aspergillus* species (with the exception of *Neosartorya fischeri*, which is the taxon closest to *A. fumigatus* [29]).

Afu*MGTC* is located on chromosome 7 between a gene coding for a rhamnosidase and a gene coding for a flavin adenine dinucleotide-dependent oxidoreductase (see Fig. S1 in the supplemental material). The gene organization around *MGTC* in *N. fisheri* was very similar (Fig. S1). In contrast, in *A. clavatus*, which does not contain an *MGTC* homolog but is taxonomically close to *A. fumigatus* and *N. fischeri*, the genes that are homologs of the genes around Afu*MGTC* are located in different chromosomes, indicating an overall rearrangement of the genomes in this area.

Phylogenetic analysis. MgtCp homologues were searched for among 62 complete genomes (http://www.ncbi.nlm.nih.gov/). A final data set of 117 amino acid positions was used for maximum-likelihood analysis (Fig. 2). Four clusters were identified. Cluster I contained bacterial species where *mgtC* has been shown to have a role in pathogenicity. Bacterial *mgtC* genes have been proposed to be acquired by HGT (5), especially in the cases of *S. enterica* serovar Typhimurium and *S. enterica* serovar Typhi, where *mgtC* was inserted into pathogenicity islands acquired by HGT.

In the phylogenetic tree, Afu*MGTC* belongs to a cluster that was not previously described (group IV), which comprises taxonomically diverse bacteria such as *Mycobacterium abscessus* and *Thermobifida fusca*. The *mgtC* genes of *T. fusca* and *M. abscessus* exhibit, respectively, 41% and 38% identity with that of *A. fumigatus*. *T. fusca* is a thermophilic bacterium that de-

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† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 20 May 2011.

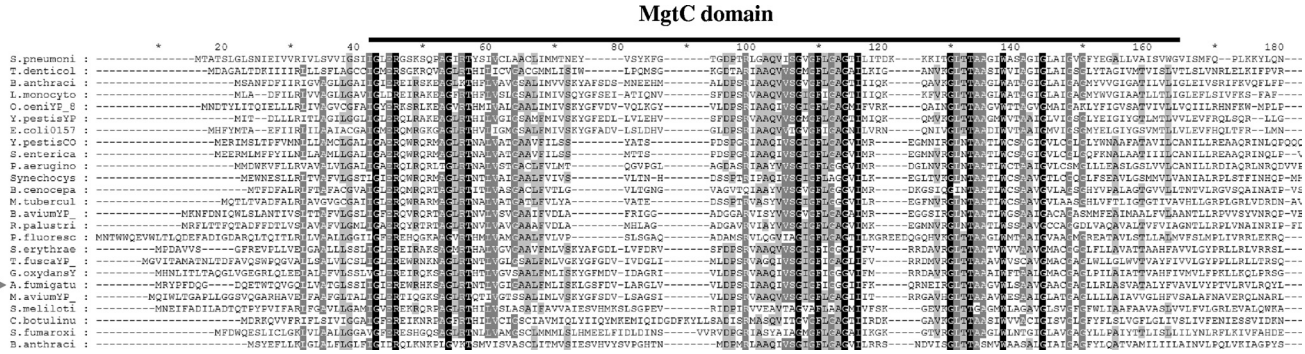

FIG. 1. Alignment of the MgtC domains of MgtC proteins. Proteins were aligned with ClustalX2, and the alignment was refined with the Genedoc program. The MgtC domain is indicated above the alignment.

grades plant cell walls in compost heaps. Interestingly, *A. fumigatus* is one of the thermophilic fungal species that are also present in compost due to its capacity to degrade decaying organic material and its thermotolerance (3). Phylogenetic group IV also contains *M. abscessus* which is a common water contaminant that is an opportunistic pathogen of cystic fibrosis patients (28) and *Saccharopolyspora erythraea* (which is close to *Saccharopolyspora rectivirgula*, one of the most important agents involved in farmer's lung disease [26]). Most of the species in clade IV have the same ecology (compost and hay).

Ripoll et al. (28) suggested that *mgtC* in *M. abscessus* could have been acquired by horizontal transfer. The specific clustering of Afu*MGTC* in a subgroup with several bacteria is consistent with HGT from a bacterium in cluster IV (2, 16). HGT has been poorly studied in fungi, and only a few studies have identified prokaryotic genes in fungi (10, 12, 15). Also, two studies of the entire genomes of *Saccharomyces cerevisiae* and *Candida parapsilosis* have been undertaken (9, 14). During the course of this study, a global analysis of HGT between prokaryotes and 60 fungal genomes was published and it reveals that the *A. fumigatus* genome contains 20 putative transferred genes, including Afu*MGTC* (22). Mallet et al. (21) have also suggested that 3.1% of the genome of *A. fumigatus* originated from bacteria, other fungi, and viruses.

Phenotypic analysis of an *mgtc* mutant. The strategy described in Fig. S2 in the supplemental material was used to produce an *A. fumigatus* strain with a nonfunctional copy of Afu*MGTC*. Psp1-MgtC was used to transform protoplasts of *A. fumigatus* CEA17Δ*ku80* (6). To complement the *mgtc* mutant, the Afu*MGTC* gene was cloned into SK+ and used with plasmid pAN8.1 (23), containing a phleomycin resistance marker, to cotransform the *mgtc* mutant. The ectopic integration of an intact copy of the Afu*MGTC* gene was verified by PCR (Fig. S2C). No growth difference between the parental, the *mgtC* mutant and the complemented *mgtc* mutant strain was observed on usual media like minimal medium, RPMI 1640 (Gibco), and Sabouraud liquid or solid medium with 3% glucose and 1% yeast extract at 37°C or 50°C. The conidial and hyphal morphology of the mutant was identical to that of the parental strain, whatever the temperature. No growth or very limited growth of the parental and mutant strains was observed in the absence of magnesium or at a low concentration of magnesium (0 or 20 μM), showing that magnesium is essential for *A. fumigatus* growth (Fig. 3A). In addition, the expression of Afu*MGTC* was not dependent on the magnesium concentration of the medium since similar reverse transcription-PCR profiles were obtained from cultures grown for 16 h at 37°C in medium supplemented with 10 μM, 50 μM, 100 μM, or 1 mM magnesium (Fig. 3B). Afu*MGTC* did not contribute to adaptation to a low-magnesium environment.

The role of Afu*MGTC* in *A. fumigatus* pathogenicity was investigated as described previously by Lambou et al. (18). No significant difference was seen in the survival rates of cohorts of 10 mice infected intranasally at 10^5 conidia/mouse with the parental strain, the *mgtc* mutant, and the complemented *mgtc* mutant strain. Survival was analyzed by the Kaplan-Meier test (chi square, 0.022) (see Fig. S3A in the supplemental material). Moreover, the conidial survival of the parental strain, the *mgtc* mutant, and the complemented *mgtc* mutant strain in the lungs of immunocompetent mice was similar, as estimated by a Student test ($P < 0.0001$) (Fig. S3B). These results indicated that Afu*MGTC* is not associated with fungal virulence.

Rang et al. (25) identified several amino acid residues involved in the loss-of-function phenotype in macrophages or in growth by complementation of the *Salmonella mgtC* mutant. E27, E84, N92, E193, and W226 were shown to be important for growth, whereas N92 and C99 were important for survival in macrophages. In *A. fumigatus*, *M. abscessus*, and *M. avium*, only E27 is conserved. These results suggested that point mutations in the sequences of members of the MgtC protein family belonging to subgroup IV are responsible for the loss of function of MgtC. Accordingly, it was not possible to complement the *Salmonella mgtC* gene with the Afu*MGTC* gene for growth in a magnesium-depleted medium, whereas other bacterial *mgtC* genes are able to complement *Salmonella* gene mutants (A. B. Blanc-Potard, data not shown; 25).

Because *A. fumigatus* growth is dependent on the presence of magnesium and Afu*MGTC* does not have any role in magnesium metabolism, other transporters that remain to be identified must play a role in magnesium metabolism in *A. fumigatus*. In eukaryotes, a family of proteins (2-TM-GxN proteins) carrying a GMN tripeptide motif between two transmembrane domains has been shown to be involved in magnesium transport (17). A member of this family is the Alr1p protein of *S. cerevisiae* (20). Several putative magnesium transporters are present in *A. fumigatus*, since we identified three genes homologous to the *S. cerevisiae ALR1* gene in the *A. fumigatus* (strain AF293) genome, AFUA_5G05830 (identity, 53%), AFUA_2G08070

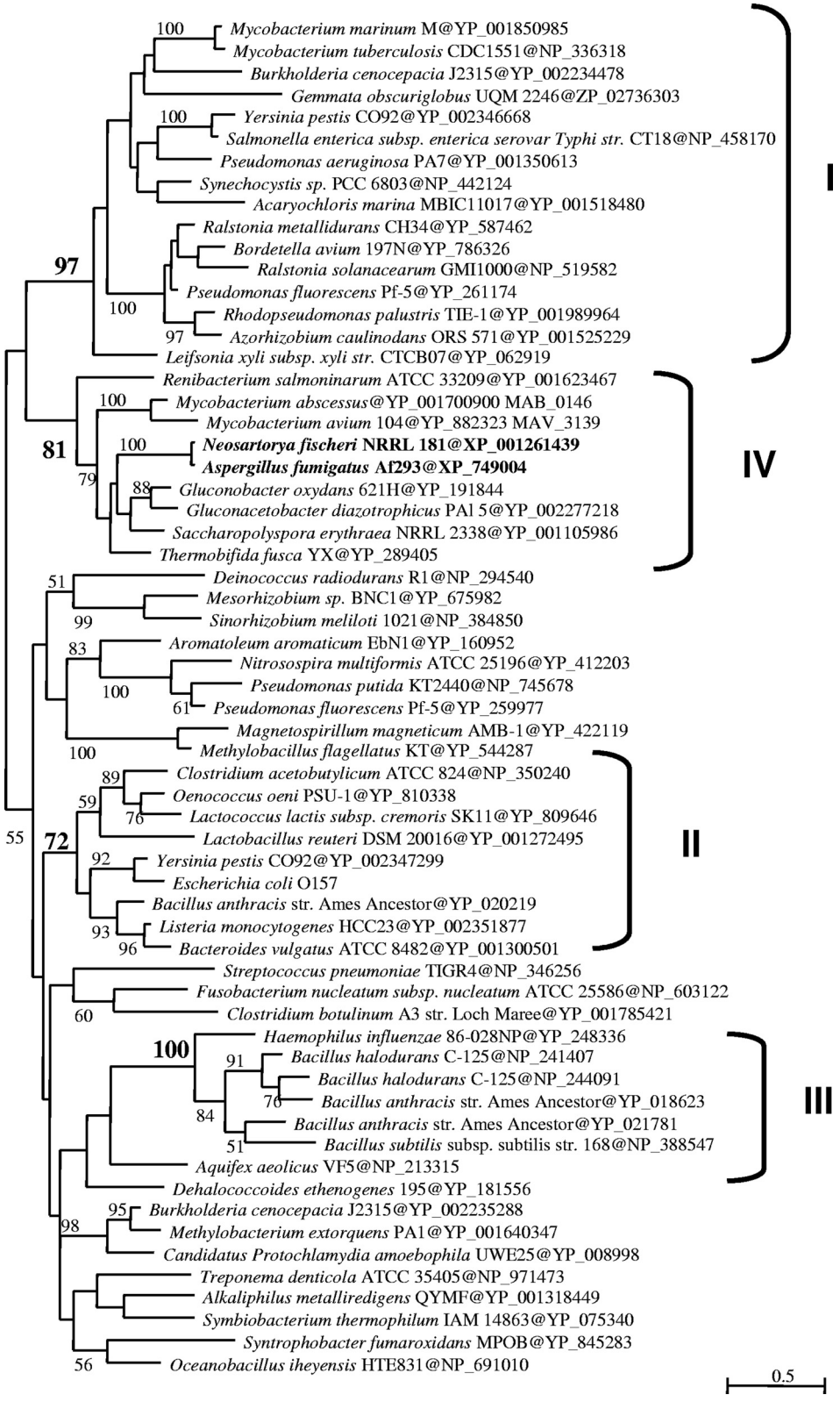

FIG. 2. Maximum-likelihood tree of MgtC orthologs. Prokaryotic and eukaryotic MgtC sequences were retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/). Values at nodes indicate statistical support calculated by nonparametric bootstrapping (only those greater than 50% are shown). The scale bar represents the average number of substitutions per site. The four clusters are indicated. Sequences were aligned by using MUSCLE 3.6 (7, 8). Sixty-two representative *MGTC* homologs were selected for phylogenetic analysis. Regions where homology was doubtful were manually removed from the alignments before phylogenetic analysis using the NET program of the MUST package (24), providing a final data set of 117 amino acid positions. Phylogenetic analysis was performed with PHYML (13), including a JTT model, a gamma correction to take into account the heterogeneity of evolutionary rates across sites (4 discrete classes of sites, an estimated alpha parameter, and an estimated proportion of invariable sites). The robustness of each branch was estimated by a nonparametric bootstrap procedure implemented in PHYML (100 replicates of the original data set and the same parameters).

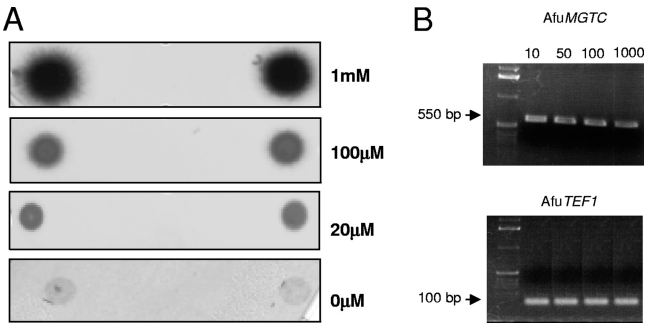

FIG. 3. (A) Growth of the parental strain and the *mgtc* mutant for 24 h at 37°C on Czapek agar medium without magnesium or supplemented with 20 μM, 100 μM, or 1,000 μM magnesium. (B) Expression levels of the Afu*MGTC* gene and the Afu*TEF1* control gene after 32 h of growth at 37°C in Czapek liquid medium supplemented with 10 μM, 50 μM, 100 μM, or 1 mM magnesium.

(identity, 46%), and AFUA_4G00930 (identity, 38%). Their functional analysis is currently under way.

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