A controllable gene-expression system for the pathogenic fungus Candida glabrata

Hironobu Nakayama, Miho Izuta, Shigehisa Nagahashi, Emi Y. Sihta, Yasuko Sato, Toshikazu Yamazaki, Mikio Arisawa and Kunio Kitada

A system for controlling gene expression was established in the pathogenic fungus Candida glabrata to elucidate the physiological functions of genes. To control the expression of the gene of interest, the C. glabrata cells were first transformed with the plasmid carrying the tetracycline repressor-transactivator fusion tetR::GAL4, then with the DNA fragment containing the controllable cassette, the tetracycline operator chimeric promoter (tetO::ScHOP1). The peptide elongation factor 3 (CgTEF3) and DNA topoisomerase II (CgTOP2) genes from C. glabrata were cloned and their expression assessed using this system. When the promoter of CgTEF3 or CgTOP2 was replaced with tetO::ScHOP1, doxycycline almost completely repressed the expression of both mRNAs, and impaired growth. Repression of the TOP2 or TEF3 gene by doxycycline also hampered the survival of C. glabrata cells in mice; in mouse kidneys the number of C. glabrata cells, in which the TOP2 or TEF3 promoter was replaced with the tetO::ScHOP1 controllable cassette, did not increase when the mice were given doxycycline. Thus, it appears that the gene repression mediated by doxycycline occurred not only in culture media but also in animals; therefore, this system can be used to elucidate the function of the gene in fungal infections and pathogenesis.

Keywords: Candida glabrata, gene expression, tetracycline-responsive element, peptide elongation factor 3, DNA topoisomerase II

INTRODUCTION

The incidence of fungal infections has been increasing, particularly in patients who are immunocompromised by HIV infection, and in patients who are receiving immunosuppressive therapy for organ transplantation or chemotherapy for cancer. Current therapies against pathogenic fungi rely mainly on two chemical groups: the polyenes, which disrupt membrane structure, and the azoles, which inhibit the synthesis of ergosterol (Gallis et al., 1990; Georgopapadakou & Walsh, 1996). However, the toxicity of the polyenes and appearance of azole-resistant strains require the development of novel antifungal drugs (Gallis et al., 1990; Rex et al., 1995).

The recent release of the complete nucleotide sequence of Saccharomyces cerevisiae has allowed us to identify all the genes in this unicellular organism (Saccharomyces Genome Database, Stanford University; The Munich Information Centre for Protein Sequences; Yeast Protein Database: Proteome); their physiological functions are currently being studied. A project to sequence the genome of the pathogenic fungus Candida albicans has been initiated. Among 6000 S. cerevisiae genes, approximately 600 have turned out to be essential for growth; deletion or disruption of any one of these genes is lethal. These essential genes are considered to be feasible antifungal targets, but such information alone is not sufficient to pinpoint the importance of the genes for infection, colonization, invasion and pathogenicity.

Unlike C. albicans, Candida glabrata always grows as a haploid yeast cell and does not form hyphae, which makes genetic manipulations easy. C. glabrata is phylogenetically similar to S. cerevisiae (Barns et al., 1991) and is an opportunistic pathogen (Aisner et al., 1976; Hickey et al., 1983). It causes mucocutaneous and systemic mycoses in immunocompromised patients who are treated with immunomodulators or anti-cancer drugs (Wingard, 1995). As seen in patients with C.
albicans infection, extensive use of topical and systemic antifungal drugs has increased the incidence of mucous-membrane colonization and the appearance of azole-resistant C. glabrata (Hitchcock et al., 1993; Newman et al., 1994). Thus, C. glabrata seems to be a feasible model organism for establishing a system applicable to the functional analysis of fungal genes for infection and virulence.

We have established a system for controlling gene expression in S. cerevisiae by using the tetracycline-responsive element (Nagahashi et al., 1997). In this study, this technique was applied to C. glabrata. The peptide elongation factor 3 (TEF3) and the DNA topoisomerase II (TOP2) genes were cloned from C. glabrata and their expression examined using this method. Because both TEF3 and TOP2 in S. cerevisiae have been shown to be essential (Sandbaken et al., 1990; Goto & Wang, 1984); they are thought to be potential targets of antifungal drugs (Triana-Alonso et al., 1995; Figgitt et al., 1989; Shen et al., 1992). Here, we report the effects of the repression of TEF3 or TOP2 of C. glabrata on its growth in media and survival in mice. A role for these genes in systemic infections is proposed.

**METHODS**

Generation of the test strain and transactivator-expressing strains. The plasmid pINTG4 (Fig. 1a) was constructed by ligating the 2.0 kb SpH1 fragment containing the repressor-transactivator fusion tetR::GAL4AD (GAL4 activation domain) from plNFGAL4 (Nagahashi et al., 1997) and the 1.5 kb KpnI fragment from pT2/Sal (Kitada et al., 1995) carrying the C. glabrata TRP1 gene (CgTRP1); pNTG4 was digested with EcoRV and used to transform the C. glabrata strains 2001HT (his3 trp1) and 2001HTU (his3 trp1 ura3) (Kitada et al., 1995). The resulting TRP" transformants, ACG4 (his3 trp1 PSch10P1::tetR::GAL4AD::TRP1) and ACG22 (his3 ura3 trp1 PSch10P1::tetR::GAL4AD::TRP1), constitutively expressed the tetR::GAL4AD fusion activator.

To generate the test strain, 97ScHIS3, p97tScHIS3U was constructed by introducing the 97t fragment, the ScHIS3 ORF and the CgURA3gene into pRS414 (Nagahashi et al., 1995). The resulting strains 2001HT (his3 trp1) and 2001HTU (his3 trp1 ura3) were transformed with the above DNA library, the complementation of the C. glabrata genomic DNA, that had been partially digested with the above PCR fragment. Genomic DNA was extracted from the C. glabrata strain ATCC 2001 as described by Rose et al. (1990). The 97t fragment was the 0.6 kb SacI/EcoRI fragment derived from p97tCC (Nagahashi et al., 1997). The ScHIS3 ORF was amplified by PCR from S. cerevisiae genomic DNA using primers H101 and H102 (Table 1), and the CgURA3 gene was amplified from C. glabrata genomic DNA using primers PCGURAS and PCGURA3 (Table 1).

Cloning the TEF3 and TOP2 genes of C. glabrata. An approximately 0.6 kb fragment of CgTEF3 was amplified from C. glabrata genomic DNA by PCR, using primers YEF3-2373 and YEF3-2996 (Table 1), which were designed based on the sequence of the S. cerevisiae YEF3 gene, encoding peptide elongation factor 3. The amplified fragment was cloned in pT7blue (Novagen). Because Southern blot analysis of C. glabrata genomic DNA using the above PCR fragment as a probe revealed that a BamHI fragment of about 9 kb strongly hybridized with the fragment, a C. glabrata genomic DNA library, which contained mainly 9 kb BamHI fragments, was generated using pBluescript II SK+ (Stratagene). The full-length TEF3 gene was identified from the library by screening with the above PCR fragment. Genomic DNA was extracted from the C. glabrata strain ATCC 2001 as described by Rose et al. (1990).

To isolate the TOP2 gene from C. glabrata, we used the S. cerevisiae temperature-sensitive mutant SD 1-4 [MATa ade1 ade2 ura3-52 top2-1{(ts)}] (DiNardo et al., 1984) for the functional screening. Another C. glabrata genomic DNA library was constructed by ligating 5–10 kb fragments of C. glabrata genomic DNA, that had been partially digested with Sau3AI, into the BamHI site of pRS416 (Stratagene). After transforming the SD 1-4 cells, with the above DNA library, the cells that grew at a non-permissive temperature (37 °C) were collected and the plasmid, which reproducibly supported the growth of SD 1-4 cells at 37 °C were recovered.
Controllable gene expression in *C. glabrata*

**Table 1.** Primers used in this study

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Generation of strains containing the *TEF3* and *TOP2* controllable cassettes. The plasmids p97CGH, p98CGH and p99CGH (Fig. 1b) were generated by replacing ScURA3 cloned in p97tCC, p98tCC or p99tCC (Nagahashi et al., 1997) with the *C. glabrata* *HIS3* (CgHIS3) gene. These plasmids comprise the *ScHOP1* promoter derivatives. p97 has both the upstream activation sequence (UAS; nt -98 to -190 in the *ScHOP1* gene) and the upstream repression sequence (URS; nt -173 to -165 in the *ScHOP1* gene), p98 carries only the URS region and p99 harbours no UAS or URS (Vershon et al., 1992; Fig. 1b). The CgHIS3 gene was amplified from *C. glabrata* genomic DNA by PCR using primers CGHISSEV and CGHIS3M (Table 1).

Region A (nt -598 to -234) or region B (nt -6 to +1215) of CgTOP2 (see Fig. 2) was amplified with PCR using primers TEF3AF and PTEF3AR, or TEF3BF and TEF3BR, respectively. For the homologous recombination, the A and B regions of CgTOP2 were cloned into the *Sacl/SmaI* sites (for region A) and *EcoRI/XhoI* sites (for region B) of p97CGH, 98CGH and 99CGH, generating p97TEF3, p98TEF3 and p99TEF3, respectively. These three plasmids were linearized with *BssHII* and used to transform ACG4 (two strains, 98TEF3 and 99TEF3), were obtained. For TOP2, the controllable cassettes carrying the A and B regions of CgTOP2 were amplified from p97CGH, p98CGH, or p99CGH by PCR using the primers CGTOPA and CGTOPB. These primers include part of the sequence of the *C. glabrata* TOP2 gene (CgTOP2). (CgTOPA contains nt -149 to -89 and CgTOPB includes nt -6 to +60 of CgTOP2) and part of p97CGH, p98CGH and p99CGH. These three controllable cassettes were used to transform ACG4; the resulting strains were designated 97TOP2, 98TOP2 and 99TOP2, respectively. All the synthetic oligonucleotides used for PCR are listed in Table 1.

**Southern and Northern blot analyses.** Ten micrograms of genomic DNA from *C. glabrata* ATCC 2001 was digested with restriction endonucleases and separated on an agarose gel. The DNA was transferred to a nylon membrane (Hybond-N, Amersham), hybridized with the radio labelled probe and visualized by autoradiography or by image analyser BAS1000 (Fujii).

Total RNA was extracted by the glass-bead lysis method (Rose et al., 1990). Approximately 10 μg total RNA was separated on an agarose gel, transferred onto a nylon membrane (Hybond-N, Amersham) and hybridized with radiolabelled probes. DNA fragments used for hybridization were the 0.5 kb *EcoRV-EcoRV* fragment of CgTOP2 (nt -6 to +394) and the 0.3 kb fragment of CgERG11 (nt -6 to +328). The signal obtained from CgERG11 was used to normalize the mRNA signals. The RNA bands that hybridized with the radiolabelled probes were visualized by autoradiography. Radiolabelling of the probe DNA was carried out by the random priming method using [α-32P]dCTP.

**Determination of cell number in vitro.** Approximately 1 × 10^8 99TEF3 cells were inoculated into YPD (1% yeast extract, 2% peptone, 2% glucose) containing the indicated concentrations of doxycycline (0–10 μg ml^-1). After 14 h at 37 °C, the OD_600 was determined. For the time-course experiments, approximately 1 × 10^6 cells of 98TOP2 or 99TEF3 were inoculated
and cultured in YPD at 37 °C with or without doxycycline (at a final concentration of 10 μg ml⁻¹); their growth was monitored by measuring OD₆₀₀ at the indicated times after the addition of doxycycline. The number of viable cells was also determined by spreading 20 μl of the diluted cultures at the indicated times after the doxycycline addition and by counting the number of colonies that had appeared after cultivation of the cells for 24 h at 37 °C.

**Determination of doxycycline concentration and C. glabrata cells in mouse organs.** Male CD-1 mice were immunocompromised by injecting them with 200 mg cyclophosphamide kg⁻¹ d⁻¹ before infection and 100 mg kg⁻¹ d⁻¹ determined by spreading 20 μl of the cyclophosphamide injection. Each mouse was intravenously inoculated with 1 x 10⁴ C. glabrata cells, and was administered doxycycline (2 mg ml⁻¹), dissolved in 5% sucrose solution, as drinking water from 6 d before the infection. On the day indicated, the mice were killed, and their liver and kidneys were removed and homogenized in 4 vols 30 mM H₂PO₄ using Sonofier 250 (Branson) at 4 °C. To determine the concentrations of doxycycline in each kidney and liver, 45 μl of 3 mM EDTA and 150 μl of CH₃CN were added to 5 μl of the 20% homogenate. The resulting diluted homogenates were centrifuged at 3500 r.p.m. for 10 min at 4 °C and 150 μl of 10 mM phosphate solution containing 1 mM EDTA was added to 50 μl of the supernatant. The concentration of doxycycline was determined by reverse-phase HPLC using a Capcell pak C18 column (5-5 mm, 46 x 150 mm; UG 120A, Shiseido) in a 20% CH₃CN solution containing 8 mM phosphate solution and 0.8 mM EDTA. To estimate the number of surviving C. glabrata cells in kidneys, the isolated kidneys were homogenized in 5 ml of distilled water, the homogenates spread on YPD plates containing penicillin G (200 units ml⁻¹) and streptomycin (200 μg ml⁻¹). After a 24 h incubation at 37 °C, the number of yeast colonies that had appeared on the plates was counted.

**RESULTS**

**Generation of a transactivator-expressing strain of C. glabrata**

To control the expression of a certain gene in C. glabrata, we applied the tetracycline-mediated transcription system, which we previously demonstrated in S. cerevisiae (Nagahashi et al., 1997). This system requires the controllable promoter, which consists of the tetO and SchOp1 promoters, and the fusion transactivator, which was generated by connecting tetR with S. cerevisiae GAL4AD. The transactivator gene was cloned in an integration plasmid, pINTG4, and then used to transform cells of C. glabrata strains 2001HT and 2001THU (Kitada et al., 1995), generating strains ACG4 and ACG22. Because SchIS3 complements a his3 null mutation of C. glabrata (Kitada et al., 1995), we first created the controllable strain 97ScHIS3 (see Methods), in which ScHIS3 was complemented with C. glabrata TEF3 for TEF3, and C5TOP2 and C3TOP2 for TOP2 genes.

**Induced repression of CgTEF3 and CgTOP2**

To study the roles of TEF3 and TOP2 in growth and infection, we replaced the promoters of CgTEF3 and CgTOP2 with 97c, 98r and 99t (Nagahashi et al., 1997) by means of homologous recombination (Fig. 2a). By the transformation of each one of the above three controllable cassettes into ACG4, the five strains 98TEF3, 99TEF3, 97TOP2, 98TOP2 and 99TOP2 were generated. 97TEF3, which contains TEF3 connected to 97t, was not obtained. The correct integration of the controllable promoters to the expected locus was confirmed by PCR using the primers C5TEF3 and C3TEF3 for TEF3, and C5TOP2 and C3TOP2 for TOP2 (Fig. 2b, c).
Controllable gene expression in C. glabrata

Table 2. Pairwise sequence identities

Values are shown as percentage amino acid identities.

(a) Peptide elongation factor 3

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(b) DNA topoisomerase II

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<td>60.2</td>
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* Qin et al. (1990).
† Di Domenico et al. (1992) and Myers et al. (1992).
‡ Ypma-Wong et al. (1992).
§ Giaever et al. (1986).
# Tsai-Pflugfelder et al. (1988) (α)
W Jenkins et al. (1992) (β).
** Wyckoff et al. (1989).

Fig. 2. Generation of controllable strains. (a) Schematic representation of the integration of the controllable promoter, tetO::SchOP1. Region A represents the 5’ flanking region of the target gene and region B indicates the 5’ end of the ORF. The DNA fragment encompassing the controllable cassette, where the CgHIS3 gene and tetO::SchOP1 are flanked by the A and B regions, was used to transform the C. glabrata strain ACG4. (b) Confirmation of the correct integration of the tetO::SchOP1 in the TEF3 locus. PCR was carried out with genomic DNA from the transformants and the wild-type ATCC 2001, with the primers for the 5’-flanking region and ORF. The expected length of the PCR products for tetO::SchOP1::TEF3 and the wild-type TEF3 are 4.6 kb and 2.6 kb respectively. Three different clones of 99TEF3 were examined. (c) Confirmation of the correct integration of tetO::SchOP1 in the TOP2 locus. The expected length of the PCR products for tetO::SchOP1-TEF3 and the wild-type TOP2 are 2.3 kb and 0.9 kb, respectively. Lanes: 4, 9, 13, DNA size markers; 1–3, three independent clones of 97TOP2; 5–8, four independent clones of 98TOP2; 10–12, three independent clones of 99TOP2; 14, wild-type. Asterisks indicate the random integration of tetO::SchOP1.
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**Fig. 3.** Effects of doxycycline on the expression of TEF3 and TOP2 and on the growth of 99TEF3 and 98TOP2. (a) Repression of TEF3 by doxycycline. Ten million 99TEF3 cells were inoculated into YPD and cultured in the absence (lanes 1–8) or presence (lanes 10–17) of 10 ng doxycycline ml⁻¹ for the indicated time. Ten micrograms of total RNA extracted from each strain was separated on an agarose gel and hybridized with the TEF3 and ERG11 probes. Lane 9 indicates the level of the TEF3 transcript in wild-type cells. (b) Repression of TOP2 by doxycycline. Ten million cells of 98TOP2 were inoculated into YPD and cultured in the presence or absence of 10 ng doxycycline ml⁻¹ for 2 h. Ten micrograms of the total RNA extracted from each strain was separated on an agarose gel and hybridized with the TOP2 and ERG11 probes. Lanes: 1; 97TOP2; 2, wild-type; 3 and 4, 98TOP2; 5, 99TOP2. (c) The number of viable 99TEF3 and 98TOP2 cells, cultured without (○) or with (■) 10 ng doxycycline ml⁻¹. (d) Morphology of 98TOP2 grown without (top) or with (bottom) 10 ng doxycycline ml⁻¹ for 12 h.

Further experiments, because they expressed the TEF3 and TOP2 mRNAs at levels similar to those observed in the wild-type cells, and their growth in the absence of doxycycline was indistinguishable from that of the wild-type (Fig. 3a, lanes 1–8 for 99TEF3, lane 9 for wild-type; and Fig. 3b, lane 1 for 97TOP2, lane 2 for wild-type, lane 3 for 98TOP2, lane 5 for 99TOP2). We used doxycycline instead of tetracycline, because it was superior to tetracycline in terms of suppressing the expression of TEF3 and TOP2 (data not shown). By Northern blotting, it was demonstrated that the TEF3 mRNA declined to an undetectable level within 30 min of the addition of doxycycline (Fig. 3a, lanes 12–17). The TOP2 mRNA also became undetectable within 2 h of doxycycline addition (Fig. 3b, lane 4). In contrast, doxycycline did not significantly affect the level of lanosterol 14-demethylase (ERG11 gene) mRNA, indicating the specificity of doxycycline to the tetO::ScHOP1 promoter (Fig. 3a, b). Seven hours after doxycycline addition, the 98TOP2 cells displayed aberrant morphology and the number of viable cells started decreasing (Fig. 3c). Most of the 98TOP2 cells became elongated and aggregated within 12 h (Fig. 3d). The growth of the 99TEF3 cells was also severely hampered by doxycycline; the number of viable cells remained very small at any time after the doxycycline addition (Fig. 3c). However, we did not observe any morphological changes in 99TEF3 cells, even when they were cultured in the presence of doxycycline. The growth impairment of 99TEF3 by doxycycline was reproducibly observed at doxycycline concentrations higher than 2 ng ml⁻¹ (Fig. 4a); the same result was obtained with 98TOP2 cells (data not shown). Together, these results demonstrate that both TEF3 and TOP2 are essential for the growth of C. glabrata, and that the depletion of TEF3 leads to immediate cell death, while the effect of TOP2 repression on viability becomes apparent after several generations.
Fig. 4. Effects of doxycycline on the survival of 99TEF3 and 98TOP2 in mouse kidneys. (a) Determination of the minimal concentrations of doxycycline required to inhibit the growth of 99TEF3. One hundred thousand 99TEF3 cells were inoculated into YPD and cultured for 14 h. OD_{660} is indicated as the percentages of those of doxycycline-untreated cells. Each value is the mean from three independent experiments. ▲, TEF3 cells; ▼, wild-type cells. (b) Concentrations of doxycycline in liver (●) and kidneys (■) of the immunocompromised mice. Each value is the mean from the three independent experiments. (c) Number of viable cells recovered from mouse kidneys. Mice that were infected with wild-type (top), 99TEF3 (middle) and 98TOP2 (bottom) were killed, and the C. glabrata cells were recovered from the kidneys. Error bars indicate standard deviation from three independent kidneys. Experiments were repeated three times. ○, Number of cells recovered from doxycycline-untreated mice; ■, number of cells recovered from doxycycline-treated mice. Error bars are not shown where the symbol is bigger than the error bar.

Effects of TEF3 and TOP2 repression on the survival of C. glabrata in mice

Next, we intended to establish the roles of TEF3 and TOP2 on the survival of C. glabrata in mice. As mentioned earlier, expression of CgTEF3 and TOP2 was almost completely repressed by 2 μg ml⁻¹ or higher concentrations of doxycycline. When doxycycline was added to the drinking water and the mice were allowed to drink freely from 6 d prior to infection, the doxycycline concentrations reached 8 μg (g kidney)⁻¹ and 10 μg (g liver)⁻¹, which are higher than the minimum concentration of doxycycline required to suppress TEF3 and TOP2 expression in vitro (Fig. 4b). Then, the number of viable C. glabrata cells in kidneys was determined by recovering the C. glabrata cells at various times after infection, because the C. glabrata cells exclusively colonized the kidneys (data not shown). The
number of viable 99TEF3 and 98TOP2 cells recovered from the kidneys was significantly lower in mice treated with doxycycline than in untreated mice. Although there was a transient increase in the number of recovered cells at 4 and 10 d post-infection, essentially 99TEF3 did not grow in mouse kidneys when doxycycline was present. The number of viable 98TOP2 cells increased up to day 4, which was followed by a rapid decline from day 10. The profiles of the doxycycline-mediated decrease in the number of viable 99TEF3 and 98TOP2 cells in the kidneys were similar to those of doxycycline-induced growth inhibition in vitro. Thus, it appears that the functions of TEF3 and TOP2 for viability are well preserved not only in cultures in vitro, but also in the organs of infected animals.

DISCUSSION

We have established a controllable gene expression system in C. glabrata. Doxycycline almost completely suppressed the expression of a gene whose transcription was under the control of the tetO::Sc HoPl promoter. Furthermore, transcriptional repression of the gene by doxycycline occurred relatively quickly. The TEF3 and TOP2 mRNA declined to an undetectable level within 2 h of the addition of doxycycline. The rapid response to doxycycline enabled us to study the roles of these genes in the survival of C. glabrata cells in animals.

By the doxycycline-induced repression, it appears that the effect of TEF3 depletion occurred much earlier than that of TOP2. Because TEF3 repression would lead to a serious defect in protein synthesis, it might cause a rapid arrest of the cell growth. 98TOP2, which expressed a lower level of the TEF3 mRNA, showed a slight growth defect even in the absence of doxycycline (data not shown). This fact implies that proper levels of TEF3 mRNA must be maintained to keep C. glabrata cells alive. The TOP2 depletion, however, affected cell growth only after several generations. This is rather an unexpected result, because Top2p is required for the completion of DNA replication and for the segregation of sister chromatids. A defect in either one of these steps may result in rapid cell death. The results obtained from this study, however, suggest that inhibition of protein synthesis more effectively causes rapid cell death than that of DNA replication.

When TOP2 was repressed, C. glabrata cells displayed an elongated morphology (Fig. 3c). A similar phenotype was also observed in the temperature-sensitive mutants of S. cerevisiae and Schizosaccharomyces pombe when they were cultured at a non-permissive temperature (DiNardo et al., 1984; Holm et al., 1985; Uemura & Yanagida, 1986). The aberrant morphology caused by a top2 mutation has been reported to be associated with the appearance of abnormal chromosomes. Therefore, the elongated morphology caused by doxycycline in 98TOP2 cells is probably a consequence of the inhibition of DNA topoisomerase II.

The effects of doxycycline on the cell growth of 99TEF3 and 98TOP2 in vitro were well reflected in animal organs. This indicates that the physiological importance of TEF3 and TOP2 is not dependent on the conditions in which C. glabrata grows. Thus, this system can be applied to other genes whose function remains to be elucidated and also to the identification of novel virulence factors.

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REFERENCES


