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(54) Title: POLYPEPTIDES INVOLVED IN CANDIDA BIOFILM FORMATION AND USES THEREOF

(57) Abstract: The present invention relates to the field of fungal biofilm formation. More specifically, the present invention relates to the identification of polypeptides and polynucleotide sequences encoding the same which are involved in Candida biofilm formation. The present invention also relates to a method for identifying compounds that affect biofilm formation and use of such compounds in compositions and methods for the prevention and the impairment of biofilm and/or treatment/prevention of candidiasis, such as those caused by Candida albicans or Candida glabrata.



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POLYPEPTIDES INVOLVED IN CANDIDA BIOFILM FORMATION AND USES THEREOF

FIELD OF THE INVENTION

5 The present invention relates to the field of fungal biofilm formation. More specifically, the present invention relates to the identification of polypeptides and polynucleotide sequences encoding the same which are involved in Candida biofilm formation. The present invention also relates to a method for identifying compounds that affect biofilm formation and use of such compounds
10 in compositions and methods for the prevention and the impairment of biofilm and/or treatment/prevention of candidiasis, such as those caused by *Candida albicans* or *Candida glabrata*.

BRIEF DESCRIPTION OF THE PRIOR ART

15 A biofilm is a community of micro-organisms attached to a surface: it contains an exopolymeric matrix and exhibits distinctive phenotypic properties (Donlan and Costerton, 2002). This type of cell development is predominant under many environmental conditions. For micro-organisms of clinical
20 importance, implanted devices (and indwelling intravascular catheters in particular) provide pathogens with surfaces on which they can adhere and subsequently form biofilms (Donlan and Costerton, 2002). Transcriptome and proteome analysis has revealed that under these growth conditions, bacterial cells adopt a metabolic profile which differs to that seen in cells growing under
25 planktonic conditions (Becker *et al.*, 2001; Whiteley *et al.*, 2001; Schembri *et al.*, 2003; Beloin *et al.*, 2004). Consequently, biofilms have been shown to be more tolerant or resistant to both host defence machinery and antimicrobial agents than free swimming cells: as a result, implant infections are difficult to treat (Baillie and Douglas, 1999; Donlan and Costerton, 2002). Since surface

attachment is a prerequisite for biofilm formation, the early stages of biofilm formation are primarily conditioned by microbial surface molecules able to bind to abiotic surfaces (referred to as adhesins). For example, in *Streptococci*, surface associated proteins such as SpaP and Fap1 have been found to function as high-affinity adhesins and play an important role in biofilm initiation and development (Bowen *et al.*, 1991; Froeliger and Pives-Taylor, 2001). Other adhesins are needed for cell-cell stabilization of the biofilm structure. For example, three glucan-binding proteins (GbpA, GbpB and GbpC) are involved in structural and functional regulation of plaque biofilms in *Streptococcus mutans* (Hazlett *et al.*, 1999).

Candida species are now ranked as the fourth most common cause of bloodstream infection in the United States and *Candida glabrata* represents the second leading cause of candidiasis after *Candida albicans* in the United States and in France (Pfaller *et al.*, 1999; Vazquez *et al.*, 1999) (F. Dromer on behalf of the French Mycoses Study Group. Initiation of an active surveillance program on yeast-related bloodstream infections in France (ASPYRIF). Abstract P9861, 4th European Congress of Clinical Microbiology and Infectious Diseases, Prague 2004). Although the majority of implant infections are caused by bacteria, the prevalence of implant infections due to fungal pathogens has dramatically increased over the last ten years.

In *C. albicans*, biofilm formation occurs in three steps. The first is the adherence of the cells to the surface, and is followed by an intermediate phase during which the microcolonies produce extracellular matrix. Finally, a maturation phase occurs, during which biofilm growth is accomplished by cells completely embedded in extracellular material (Chandra *et al.*, 2001). *C. glabrata* has also been shown to be capable of forming a biofilm on a range of plastic surfaces, although the presence of extracellular matrix has not been demonstrated (Nikawa *et al.*, 1997; Kumamoto, 2002; Shin *et al.*, 2002). Various adhesin classes have been identified in *C. albicans*, and some have been shown to be involved in biofilm formation (Sundstrom, 1999). For instance, *ALS1* expression is induced in biofilms (Chandra *et al.*, 2001; García-Sánchez *et al.*, 2004; Green *et al.*, 2004)., and *ALS3* is necessary for wild-type biofilm

formation in *C. albicans* (Zhao, X., Daniels, J.K., Oh, S., Green, C.B., Soll, D.R. and Hoyer L.L. Abstract 33C, 7th ASM conference on Candida and candidiasis, 2004). In *C. glabrata*, five adhesin encoding genes (*EPA1*, *EPA2*, *EPA3*, *EPA4* and *EPA5*) have been previously identified (Cormack *et al.*, 1999; De las Penas *et al.*, 2003), although none has been studied for its involvement in biofilm formation. *EPA1* is mainly expressed during the exponential growth phase: it encodes a Ca^{2+} -dependent lectin which recognizes N-acetyl lactosamine and which is necessary for the mediation of yeast cell adherence to cultured human epithelial cells (Cormack *et al.*, 1999). The five *EPA* genes are organized into two clusters located within sub-telomeric regions and consequently, *EPA2* to *EPA5* are not expressed in wild type *C. glabrata* under all tested conditions (De las Penas *et al.*, 2003).

In view of the above, there is a need to provide new polypeptides as well as polynucleotides encoding such polypeptides involved in regulation of Candida biofilm formation. There is also a need to provide for new compositions comprising inhibitors of such polypeptides in the treatment and impairment of biofilm formation in animals.

SUMMARY OF THE INVENTION

The present invention satisfies at least one of the above-mentioned needs.

More specifically, an object of the invention is to provide a method of screening for a compound that affects expression of a gene involved in biofilm formation of a Candida strain, said gene being selected from the group consisting of *EPA6*, *EPA7* and *YAK1*, the method comprising the steps of:

- providing a Candida biofilm with an initial expression level for said gene;
- contacting said biofilm with at least one compound to be tested;
- evaluating expression of the gene; and

- identifying the compound that inhibits the initial expression level of said gene in the biofilm.

Another object of the invention concerns a compound that affects biofilm formation of a *Candida* strain obtained by the method as defined above. A
5 related object is to provide a composition for preventing and/or treating candidiasis, comprising a compound of the invention and an acceptable carrier.

A further object is to provide a method for an early diagnostic of a *Candida* strain forming a biofilm, comprising the steps of:

- a) obtaining a cell sample of said *Candida* strain;
- 10 b) measuring the mRNA level of EPA6, EPA7 and/or YAK1 genes in said cell sample; and

c) measuring the mRNA level of said gene in a control *Candida* cell culture at early planktonic growth,
wherein overexpression of EPA6, EPA7 and/or YAK1 genes in said cell sample
15 compared to the control cell culture, is indicative of the presence of a *Candida* strain forming a biofilm.

Alternatively, a further object is to provide a method for an early in vitro diagnostic of a *Candida* strain forming a biofilm, comprising the steps of:

- a) measuring the mRNA level of EPA6, EPA7 and/or YAK1 genes in a
20 cell sample of said *Candida* strain; and

b) measuring the mRNA level of said gene in a control *Candida* cell culture at early planktonic growth,
wherein overexpression of EPA6, EPA7 and/or YAK1 genes in said cell sample compared to the control cell culture, is indicative of the presence of a *Candida*
25 strain forming a biofilm.

Yet, another object is to provide a method for detecting the presence or absence of a *Candida* biofilm in a sample, comprising the steps of:

- 5
- a) contacting the sample with at least one molecule that specifically recognizes a *Candida* Yak1, Epa7 or Epa6 polypeptide for a time and under conditions sufficient to form a complex; and
 - b) detecting the presence or absence of the complex formed in a).

10 Another object of the invention is to provide a method for preventing and/or treating candidiasis in an animal, the method comprising the step of administering to the animal, a therapeutically effective amount of a composition as defined above.

15 Furthermore, the present invention is concerned with the use of the compound of the invention in the preparation of a medicament for preventing or treating candidiasis. The present invention is further concerned with the use of at least one *Candida* protein selected from the group consisting of Epa6, Epa7, Sir4, Rif1 and Yak1 or at least one gene encoding said protein as a target for identifying a compound capable of impairing biofilm formation of a *Candida* strain.

Yet, another embodiment of the invention is to provide a kit for the detection of the presence or absence of a *Candida* biofilm, comprising:

- 20
- at least one antibody that specifically binds to a *Candida* Yak1, Epa7 or Epa6 protein;
 - a reagent to detect polypeptide-antibody immune complex;
 - optionally a biological reference sample lacking Yak1, Epa7 or Epa6 polypeptides; and
- 25
- optionally a comparison sample comprising Yak1, Epa7 or Epa6 polypeptides which specifically bind to said antibody;

wherein said antibody, reagent, biological reference sample, and comparison sample are present in an amount sufficient to perform said detection.

Yet, a further embodiment of the invention is to provide a kit for the early
5 diagnostic of a Candida strain forming a biofilm, comprising:

- specific primers for Candida EPA6, EPA7 and/or YAK1 genes;
- specific probes for Candida EPA6, EPA7 and/or YAK1 genes;
- reagents for detecting the mRNA level of EPA6, EPA7 and/or YAK1 genes;
- 10 - a control sample of Candida cell culture at an early planktonic growth phase;
- optionally a biological reference sample lacking Epa6, Epa7 and/or Yak1 polynucleotides; and
- optionally a comparison sample comprising EPA6, EPA7 and/or YAK1
15 polynucleotides which specifically hybridize with said primers or probes;

wherein said primers, probes, reagents, biological reference sample, and comparison sample are present in an amount sufficient to perform said determination.

The present invention also provides a method for selecting a biofilm
20 formation inhibitor of a Candida strain acting through a Yak1 dependent pathway, comprising the following steps:

a) contacting a compound with a biofilm of a wild type Candida strain and determining if it inhibits biofilm formation of said Candida strain;

b) contacting the compound selected in a) with a biofilm of a *sir4* mutant
25 Candida strain and determining if it inhibits biofilm formation of said Candida strain;

c) selecting the compound identified in a) if said compound inhibits the formation of biofilm of the wild-type *Candida* strain and do not inhibit the formation of biofilm of the *sir4* mutant *Candida* strain.

5 The present invention is also concerned with an *in vitro* screening method for an inhibitor of the activity of a Yak1 polypeptide of *Candida*, comprising the steps of :

a) contacting a purified or isolated Yak1 recombinant polypeptide or a fragment thereof retaining the kinase activity with a candidate compound;

10 b) measuring the capacity of said candidate compound to inhibit the kinase activity of the Yak1 polypeptide or fragment thereof, said kinase activity being measured by a fluorescent and/or luminescent kinase assay.

15 The present invention is also concerned with a purified or isolated polypeptide involved in *Candida* biofilm formation comprising an amino acid sequence substantially identical to SEQ ID NO: 1, and a purified polynucleotide encoding said purified polypeptide.

The present invention is further concerned with a purified or isolated purified polypeptide having a kinase activity sensitive to N-MPP1 comprising the amino acid sequence SEQ ID NO: 9.

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BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Biofilm formed by *C. glabrata* strain BG2 on a plastic slide. Biofilm was formed in YPD medium at 37°C on the surface of a Thermanox slide. At the highest magnification, residual extracellular polymeric material is visible. (A) magnification X200; (B) magnification X2000; (C) magnification X20000.

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Figure 2. *C. glabrata* cells are more adherent in the stationary phase. (A) Influence of the growth phase on biofilm formation by *C. glabrata* strain BG2. The cells were grown on YPD medium and the OD₆₀₀ of the culture was

followed. At different stage of growth ($OD_{600} = 0.5, 2, 4, 5.4$ and 12.5) an aliquot was recovered. The cells were washed, resuspended in YPD medium ($OD_{600} = 1$). The relationship between the OD_{600} and the number of cell in the suspension was not affected by the stage of growth (2.7 ± 0.3 cell/ml for $OD_{600} = 1$). The cells were then distributed into microtiter plate wells. After 24h of growth at 37°C , less-adherent cells were eliminated by serial washes and XTT reduction was measured. (B) Kinetics of XTT reduction according to the time of incubation of adherent cells with the XTT.

Figure 3. *C. glabrata* Biofilm mutants. (A) Biofilm formed by *C. glabrata* mutant strains on a plastic slide. Biofilms were formed in SC medium at 37°C on the surface of a Thermanox slide. The Biofilm- strains produced smaller structures at the plastic surface whereas Biofilm++ strains produced very large and complex structures (B) Strains were grown under biofilm conditions in 96 well plates in SC medium at 37°C , with less-adherent cells being eliminated by serial washes. Adherent cells were examined and photographed using an inverted microscope (Zeiss Axiovert 200M) at two different magnifications. Wells containing wildtype strain (BG2), Biofilm- (CG129) and Biofilm++ (CG137) mutant strains are shown.

Figure 4. *EPA6* and *EPA7* in *Candida glabrata*. (A) Schematic representations of the *EPA6-7* gene and the disruption strategy. (B) Southern-blot analysis of the DNA digested by *Clal* or *HindIII* and hybridized with the *EPA6-EPA7* specific probe.

Figure 5. Epa6p (Epa6 protein) influences biofilm formation in *C. glabrata* (A) Biofilm formation by the CG122 (*epa6-1*), CG164 (*epa6Δ*), CG129 (*yak1*) and BG570 (*epa1-5Δ*) strains as monitored by an XTT reduction assay). 100% Ac corresponds to the amount of the XTT reduced by the wild type strain (BG2). The reported values are the means $\pm$ SD of three independent experiments. (B) Transcription of *EPA6* and *EPA7* as assessed by RT-PCR. Cells were grown were grown under planktonic (late stationary phase of growth) or biofilm growth

conditions (see Examples Section). *ACT1* was used as a control. (C) Transcription of *EPA6* at different stages of the planktonic growth on SC medium at 37°C as compare to biofilm growth. Cells were grown under planktonic growth and an aliquot was recovered after (1) 2h of growth ($DO_{600} = 0.1$); (2) 4h of growth ($DO_{600} = 0.24$); (3) 6h of growth ($DO_{600} = 0.72$); (4) 8h of growth ($DO_{600} = 2.1$); (5) 10h of growth ($DO_{600} = 4.9$); (6) 24h of growth ($DO_{600} = 7.7$); (7) 48h of growth ($DO_{600} = 7.42$); (8) Cells were grown under biofilm growth conditions for 24h using the 96 well plate model (see Examples Section). *ACT1* was used as a control.

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Figure 6. Variation of *URA3* gene expression when inserted at the *EPA6* locus. Each strain of *C. glabrata* was grown on YPD medium at 37°C under agitation. The cells were then washed with sterile water: serial dilutions of cells (10^7 , 10^6 and 10^5) were spotted on YPD, SD and FOA media and incubated at 37°C for 24h.

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Figure 7. Biofilm formation is regulated by sub-telomeric silencing in *C. glabrata*. (A) Biofilm formation by BG2, CG145 (*sir4Δ*), CG149 (*rif1Δ*), BG676 (*sir3Δ*), BG592 (*rap1-21*) and CG170 (*sir4Δepa6Δ*) strains was monitored by an XTT reduction assay. 100% Ac correspond to the amount of the XTT reduced by the wild type strain (BG2). The reported values are the means $\pm$ SD of three independent experiments. (B) Transcription of *EPA6* and *EPA7* as assessed by RT-PCR. BG2, CG145 (*sir4Δ*), CG149 (*rif1Δ*) and CG170 (*sir4Δ epa6Δ*) strains were grown under planktonic growth conditions (late stationary phase of growth) (see Examples Section). *ACT1* was used as control.

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Figure 8. The biofilm signal and Yak1p (Yak1 protein) act through a sub-telomeric silencing-dependent Mpk1p-independent pathway (A) Biofilm formation by BG2, CG145 (*sir4Δ*), CG172 (*sir4Δyak1*) and CG174 (*mpk1Δ*) strains was monitored by XTT reduction assay. 100% Ac correspond to the amount of the XTT reduced by the wild type strain (BG2). (B) Transcription of *EPA6* and *EPA7* as assessed by RT-PCR. BG2, CG145 (*sir4Δ*), CG149 (*rif1Δ*),

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BG676 (*sir3Δ*), CG172 (*sir4Δ yak1*) and CG174 (*mpk1Δ*) strains were grown under planktonic (late stationary phase of growth) or biofilm growth conditions in the 96-well plate model (see Examples Section). *ACT1* was used as control.

5 **Figure 9.** Model for *EPA6-EPA7* regulation in *C. glabrata*.

Figure 10. *YAK1* is necessary to the formation of biofilm in *Candida albicans*.

Strains *yak1D* of *C. albicans* do not possess growth defects on complete media (YPD) but their filament process is altered (Lee medium). These strains do not
10 produce biofilm microfermentor or tubular models.

Figure 11. Restoration of the capacity of biofilm formation of double mutant $\Delta yak1/\Delta yak1$ of *C. albicans* after the introduction of wild-type *yak1* gene.

15 **Figure 12.** *YAK1* analogue sensitive kinase allele in *C. albicans*. A copy of the *yak1* gene with a mutation (Phe507 : Ala) was introduced in the $\Delta yak1/\Delta yak1$ strain to create a *YAK1* analogue sensitive kinase allele with an enlarged binding pocket.

20 **Figure 13.** Sensitivity of Yak (Phe507 : Ala) of *C. albicans* to 1-N-MPP1. Hyphal differentiation in LEE medium of the condition allele strain.

Figure 14. *Candida albicans* Yak1 amino acid sequence (SEQ ID NO: 1).

25 **Figure 15.** *Candida glabrata* Epa6 amino acid sequence (SEQ ID NO: 2).

Figure 16. *Candida glabrata* Epa7 amino acid sequence (SEQ ID NO: 3).

Figure 17. Amino acid sequence of a *Candida albicans* Yak1 functional
30 derivative (SEQ ID NO: 4).

Figure 18. Nucleic acid sequence encoding the *Candida albicans* Yak1 amino acid sequence of figure 14 (SEQ ID NO: 5).

5 **Figure 19.** Nucleic acid sequence encoding the *Candida glabrata* Epa6 amino acid sequence of figure 15 (SEQ ID NO: 6).

Figure 20. Nucleic acid sequence encoding the *Candida glabrata* Epa7 amino acid sequence of figure 16 (SEQ ID NO: 7).

10 **Figure 21.** Nucleic acid sequence encoding the *Candida albicans* Yak1 amino acid sequence derivative of figure 17 (SEQ ID NO: 8).

Figure 22. Amino acid sequence of *Candida albicans* N-MPP1-sensitive mutant Yak1 protein (SEQ ID NO: 9)

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Figure 23. *Candida glabrata* Yak1 amino acid sequence (SEQ ID NO:10).

Figure 24. Nucleic sequence encoding the *Candida glabrata* Yak1 amino acid sequence of Figure 23 (SEQ ID NO: 11).

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DETAILED DESCRIPTION OF THE INVENTION

The inventors have surprisingly found *Candida* genes and encoded polypeptides (or proteins) that are implicated in the formation of *Candida* biofilm. In this connection, the present invention specifically relates to the use of said polypeptides in methods for identifying compounds that affect *Candida* biofilm formation. Such compounds shall be used in compositions and methods for the impairment and the prevention of *Candida* biofilm and/or for the treatment/prevention of candidiasis, such as those caused by *Candida albicans* or *Candida glabrata*. The present invention further relates to detection methods of *Candida* biofilms.

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1. Methods of screening/selection and compounds

In a first embodiment, the present invention is thus concerned with the use of at least one Candida protein selected from the group consisting of Epa6, Epa7, Sir4, Rif1 and Yak1 or at least one gene encoding said protein as a target for identifying a compound capable of impairing biofilm formation of a Candida strain. The present invention is also concerned with the use of such a compound in the preparation of a medicament for preventing or treating candidiasis.

As used herein, the expression "biofilm formation" refers to either already formed biofilm or to a Candida cell culture which is in conditions that induce or facilitate the formation of a biofilm.

In a related aspect, the invention is particularly concerned with a method of screening for a compound that affects biofilm formation of a Candida strain such as *Candida glabrata* or *Candida albicans*. More specifically, the method contemplated by the present invention is for the screening of a compound that affects expression of a gene involved in biofilm formation of a Candida strain, wherein the genes are selected from the group consisting of EPA6, EPA7, SIR4, RIF1 and YAK1. The method of the invention comprises the steps of:

- providing a Candida biofilm with an initial expression level for said gene;
- contacting said biofilm with at least one compound to be tested;
- evaluating expression of the gene; and
- identifying the compound that inhibits the initial expression level of said gene in the biofilm.

The screening method of the invention may further comprises a step of evaluating inhibition of biofilm formation by the compound.

According to a preferred embodiment of the screening method of the invention, the compound inhibits expression of gene EPA6, EPA7 and/or YAK1

by down-regulating the transcription of the gene EPA6, EPA7 and/or YAK1, or by down-regulating the translation of mRNA in a protein encoded by the gene EPA6, EPA7 and/or YAK1. More preferably, the down-regulation of transcription is evaluated by measuring the mRNA level whereas the the down-regulation of translation is evaluated by measuring the protein level. The methods for measuring the above-mentioned levels are well within the common knowledge of a person in the art and will thus not be further detailed.

It will be further be noted that in the case where the Candida strain preferably consists of *Candida glabrata*, the gene whose expression is evaluated is preferably EPA6, EPA7 or YAK1, whereas where the Candida strain preferably consists of *Candida albicans*, the gene whose expression is evaluated is preferably YAK1.

In another embodiment, the present invention concerns a compound obtained by the above-mentioned screening method of the invention. Such a compound has the capacity of affecting biofilm formation of a Candida strain, such as *Candida albicans* or *Candida glabrata*. It will be understood that the compound of the invention affects biofilm formation (for instance, by inhibiting or preventing biofilm formation or its development) preferably by decreasing the expression of the polypeptide and/or the polynucleotide contemplated by the present invention, in said fungus.

As it may be appreciated, the compound of the invention finds a particular use as an anti-fungal agent.

According to another embodiment of the invention, it is provided a method for an early diagnostic of a Candida strain forming a biofilm, comprising the steps of:

- a) obtaining a cell sample of said Candida strain;
- b) measuring the mRNA level of EPA6, EPA7 and/or YAK1 genes in said cell sample; and

c) measuring the mRNA level of said gene in a control *Candida* cell culture at early planktonic growth,

wherein overexpression of EPA6, EPA7 and/or YAK1 genes in said cell sample compared to the control cell culture, is indicative of the presence of a *Candida* strain forming a biofilm.

5

Alternatively, it is provided a method for an early in vitro diagnostic of a *Candida* strain forming a biofilm, comprising the steps of:

a) measuring the mRNA level of EPA6, EPA7 and/or YAK1 genes in a cell sample of said *Candida* strain; and

b) measuring the mRNA level of said gene in a control *Candida* cell culture at early planktonic growth,

10

wherein overexpression of EPA6, EPA7 and/or YAK1 genes in said cell sample compared to the control cell culture, is indicative of the presence of a *Candida* strain forming a biofilm.

It will be further be noted that in the case where the *Candida* strain consists of *Candida glabrata*, the gene whose mRNA level is measured is EPA6, EPA7 and/or YAK1, whereas where the *Candida* strain consists of *Candida albicans*, the gene whose mRNA level is measured is YAK1.

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Although the mRNA level is preferably quantified by RT-PCR, it will be understood that any method known to be suitable for the quantification of the level of mRNA, such as a hybridization assay, is within the scope of the present invention.

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According to a further object of the invention, there is provided a method for selecting a biofilm formation inhibitor of a *Candida* strain acting through a Yak1 dependent pathway, comprising the following steps:

25

a) contacting a compound with a biofilm of a wild type *Candida* strain, such as *Candida glabrata*, and determining if it inhibits biofilm formation of said *Candida* strain;

5 b) contacting the compound selected in a) with a biofilm of a *sir4* mutant *Candida* strain, such as *Candida glabrata*, and determining if it inhibits biofilm formation of said *Candida* strain;

c) selecting the compound identified in a) if said compound inhibits the formation of biofilm of the wild-type *Candida* strain and do not inhibit the formation of biofilm of the *sir4* mutant *Candida* strain.

10 Preferably, the above mentioned method for selecting a biofilm formation inhibitor may advantageously used the *sir4* mutant *Candida glabrata* strain Cg145 of the present invention, which was filed at the C.N.C.M. (Institut Pasteur, 25 rue du docteur Roux, 75724 Paris cedex 15, France) on November 30, 2005 under the reference number I-3532.

15 There is also provided an *in vitro* screening method for an inhibitor of the activity of a Yak1 polypeptide of *Candida*, comprising the steps of :

a) contacting a purified or isolated Yak1 recombinant polypeptide or a fragment thereof retaining the kinase activity with a candidate compound;

20 b) measuring the capacity of said candidate compound to inhibit the kinase activity of the Yak1 polypeptide or fragment thereof, said kinase activity being measured by a fluorescent and/or luminescent kinase assay. Preferably, the Yak1 recombinant polypeptide has an amino acid sequence substantially identical to SEQ ID NO: 1, 4 or 10.

25 As one skilled in the art may appreciate, the selecting method of the invention may be combined with the *in vitro* screening method of the invention for selecting a biofilm formation inhibitor of a *Candida* strain acting through a Yak1 dependent pathway.

In another embodiment, the present invention concerns a compound obtained by the above-mentioned in vitro screening and/or selecting methods of the invention. Such a compound has the capacity of affecting biofilm formation of a *Candida* strain, such as *Candida albicans* or *Candida glabrata*.

5 According to a further object of the invention, there is provided a method for detecting the presence or absence of a *Candida* biofilm in a sample, comprising the steps of:

- 10 a) contacting the sample with at least one molecule that specifically recognizes a *Candida* Yak1, Epa7 or Epa6 polypeptide for a time and under conditions sufficient to form a complex; and
- b) detecting the presence or absence of the complex formed in a).

15 It will be further be noted that in the case where the *Candida* strain is preferably *Candida glabrata*, the at least one molecule preferably specifically recognises the Yak1, Epa7 or Epa6 polypeptide, whereas where the *Candida* strain is preferably *Candida albicans*, the at least one molecule preferably specifically recognises the Yak1 polypeptide.

20 As used herein, the term "sample" refers to a variety of sample types obtained from an individual and can be used in a detection assay or method. The definition encompasses blood and other liquid samples of biological origin, solid tissue samples such as a biopsy specimen or tissue cultures or cells derived therefrom.

25 According to preferred embodiments of the biofilm detection method of the invention and of the in vitro screening method of the invention, the Yak1, Epa6 and Epa7 polypeptides comprise respectively an amino acid sequence substantially identical to SEQ ID NO: 1 (and 10 for Yak1), 2 and 3 or functional derivatives thereof. By "substantially identical", it will be understood that the *Candida* Yak1, Epa6 or Epa7 polypeptides contemplated by the present invention preferably have an amino acid sequence having at least 80% identity, or even preferably 85% identity, or even more preferably 95% identity to part or

all respectively of SEQ ID NO:1 (and SEQ ID NO: 10 for Yak1), SEQ ID NO:2 and SEQ ID NO:3, or functional derivatives thereof.

Yet, more preferably, the Candida Yak1, Epa6 and Epa7 polypeptides comprise an amino acid sequence having 100% identity respectively with SEQ
5 ID NO:1 (and SEQ ID NO: 10 for Yak1), SEQ ID NO:2 and SEQ ID NO:3.

As used herein, the terms "polypeptide" and "protein" are used interchangeably to denote an amino acid polymer or a set of two or more interacting or bound amino acid polymers.

In this connection, the present invention further concerns a purified or
10 isolated polypeptide involved in Candida biofilm formation. Such a polypeptide comprises an amino acid sequence substantially identical to SEQ ID NO: 1. The present invention is also concerned with a purified or isolated polynucleotide encoding the polypeptide contemplated by the invention. Such a polynucleotide preferably comprises the nucleic acid sequence SEQ ID NO: 5.

15 The invention further concerns a purified or isolated polypeptide having a kinase activity sensitive to N-MPP1 comprising the amino acid sequence SEQ ID NO: 9.

By "isolated" is meant, when referring to a polypeptide, that the indicated molecule is separate and discrete from the whole organism with which the
20 molecule is found in nature or is present in the substantial absence of other biological macro-molecules of the same type. The term "isolated" with respect to a polynucleotide is a nucleic acid molecule devoid, in whole or part, of sequences normally associated with it in nature; or a sequence, as it exists in nature, but having heterologous sequences in association therewith; or a
25 molecule disassociated from the chromosome.

Broadly defined, the terms "purified polypeptide" or "purified polynucleotide" refer to polypeptides or polynucleotides that are sufficiently free of other proteins or polynucleotide, or carbohydrates, and lipids with which they are naturally associated. The polypeptide or polynucleotide may be purified by

any process by which the protein or polynucleotide are separated from other elements or compounds on the basis for instance, of charge, molecular size, or binding affinity.

5 A "functional derivative", as is generally understood and used herein, refers to a protein/peptide sequence that possesses a functional biological activity that is substantially similar to the biological activity of the whole protein/peptide sequence. In other words, it refers to a polypeptide or fragment(s) thereof that substantially retain(s) the capacity of being involved in fungal biofilm formation. A functional derivative of a protein/peptide may or may not contain post-translational modifications such as covalently linked
10 carbohydrates, if such modification is not necessary for the performance of a specific function. The term "functional derivative" is meant to encompass the "fragments", "segments", "variants", "analogs" or "chemical derivatives" of a protein/peptide. As used herein, a protein/peptide is said to be a "chemical
15 derivative" of another protein/peptide when it contains additional chemical moieties not normally part of the protein/peptide, said moieties being added by using techniques well known in the art. Such moieties may improve the protein/peptide solubility, absorption, bioavailability, biological half life, and the like. Any undesirable toxicity and side-effects of the protein/peptide may be
20 attenuated and even eliminated by using such moieties. For instance, such a derivative with regards to Yak1 protein is a polypeptide having an amino acid sequence as shown in SEQ ID NO: 4 and comprising the sequence coding for the kinase activity.

25 One can use a program such as the CLUSTAL program to compare amino acid sequences. This program compares amino acid sequences and finds the optimal alignment by inserting spaces in either sequence as appropriate. It is possible to calculate amino acid identity or homology for an optimal alignment. A program like BLASTp will align the longest stretch of similar sequences and assign a value to the fit. It is thus possible to obtain a
30 comparison where several regions of similarity are found, each having a

different score. Both types of identity analysis are contemplated in the present invention.

Moreover and according to other preferred embodiments, the preferred Yak1, Epa6 and Epa7 amino acid sequences used in accordance with the
5 biofilm detection method of the invention are preferably encoded respectively by a nucleotide sequence substantially identical to SEQ ID NOS: 5 (and 11 for Yak1), 6 and 7, or functional fragments thereof.

By "substantially identical", it will be understood that the nucleotide
10 sequence contemplated by the present invention preferably has a nucleic acid sequence which is at least 65% identical, more particularly 80% identical and even more particularly 95% identical to part or all of any one of SEQ ID NOS 5, 11, 6 or 7 or functional fragments thereof.

A "functional fragment", as is generally understood and used herein,
15 refers to a nucleic acid sequence that encodes for a functional biological activity that is substantially similar to the biological activity of the whole nucleic acid sequence. In other words, it refers to a nucleic acid that substantially retains the capacity of encoding a polypeptide involved in Candida biofilm formation.

The term "fragment", as used herein, refers to a polynucleotide sequence
20 (e.g., cDNA) which is an isolated portion of the subject nucleic acid constructed artificially (e.g., by chemical synthesis) or by cleaving a natural product into multiple pieces, using restriction endonucleases or mechanical shearing, or a portion of a nucleic acid synthesized by PCR, DNA polymerase or any other polymerizing technique well known in the art, or expressed in a host cell by recombinant nucleic acid technology well known to one of skill in the art. For
25 instance, such a fragment with respect to YAK1 gene is a polynucleotide having a nucleic acid sequence as shown in SEQ ID NO 8.

Amino acid or nucleotide sequence "identity" and "similarity" are determined from an optimal global alignment between the two sequences being compared. An optimal global alignment is achieved using, for example, the

Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, J. Mol. Biol. 48:443-453). "Identity" means that an amino acid or nucleotide at a particular position in a first polypeptide or polynucleotide is identical to a corresponding amino acid or nucleotide in a second polypeptide or polynucleotide that is in an optimal global alignment with the first polypeptide or polynucleotide. In contrast to identity, "similarity" encompasses amino acids that are conservative substitutions. A "conservative" substitution is any substitution that has a positive score in the blosum62 substitution matrix (Hentikoff and Hentikoff, 1992, Proc. Natl. Acad. Sci. USA 89: 10915-10919). By the statement "sequence A is n% similar to sequence B", it is meant that n% of the positions of an optimal global alignment between sequences A and B consists of identical residues or nucleotides and conservative substitutions. By the statement "sequence A is n% identical to sequence B", it is meant that n% of the positions of an optimal global alignment between sequences A and B consists of identical residues or nucleotides.

As one skilled in the art may appreciate, the molecule preferably used in connection with the biofilm detection method of the invention is an antibody that specifically binds to said Yak1, Epa6 or Epa7 polypeptides or functional derivative thereof. More preferably, the antibody is a monoclonal antibody.

As used herein, the term "specifically binds to" refers to antibodies that bind with a relatively high affinity to one or more epitopes of a protein of interest, but which do not substantially recognize and bind to molecules other than the one(s) of interest. As used herein, the term "relatively high affinity" means a binding affinity between the antibody and the protein of interest of at least 10^{-6} M, and preferably of at least about 10^{-7} M and even more preferably 10^{-8} M to 10^{-10} M. Determination of such affinity is preferably conducted under standard competitive binding immunoassay conditions which is common knowledge to one skilled in the art.

As used herein, the term "antibody" refers to a glycoprotein produced by lymphoid cells in response to a stimulation with an immunogen. Antibodies possess the ability to react *in vitro* and *in vivo* specifically and selectively with

an antigenic determinant or epitope eliciting their production or with an antigenic determinant closely related to the homologous antigen. The term "antibody" is meant to encompass constructions using the binding (variable) region of such an antibody, and other antibody modifications. Thus, an antibody useful in the method of the invention may comprise a whole antibody, an antibody fragment, a polyfunctional antibody aggregate, or in general a substance comprising one or more specific binding sites from an antibody. The antibody fragment may be a fragment such as an Fv, Fab or F(ab')₂ fragment or a derivative thereof, such as a single chain Fv fragment. The antibody or antibody fragment may be non-recombinant, recombinant or humanized. The antibody may be of an immunoglobulin isotype, e.g., IgG, IgM, and so forth. In addition, an aggregate, polymer, derivative and conjugate of an immunoglobulin or a fragment thereof can be used where appropriate.

2. Methods of use and compositions

The compounds obtained by the screening/selecting methods of the invention may be used in many ways in the prevention and/or treatment of candidiasis, preferably caused by the formation of biofilm by *Candida* strains.

In this connection, another embodiment of the present invention relates to a composition for preventing and/or treating candidiasis, and more particularly for preventing and/or impairing the formation of biofilm by *Candida* strains. The composition of the present invention advantageously comprises a compound of the invention and an acceptable carrier.

As used herein, the term "impairing" refers to a process by which the formation or development of a *Candida* biofilm, such as one of *C. albicans* or *glabatra*, is affected or completely destroyed. As used herein, the term "preventing" when referring to biofilm formation, refers to a process by which with the formation or development of a *Candida* biofilm, such as *C. albicans* or *glabatra*, is obstructed or delayed.

When referring in general to candidiasis, the term "preventing or prevention" refers to a process by which the symptoms of candidiasis is obstructed or delayed. By the term "treating" is intended, for the purposes of this invention, that the symptoms of candidiasis be ameliorated or completely eliminated.

As used herein, the expression "an acceptable carrier" means a vehicle for containing the compounds obtained by the method of the invention that can be administered to a animal host without adverse effects. Suitable carriers known in the art include, but are not limited to, gold particles, sterile water, saline, glucose, dextrose, or buffered solutions. Carriers may include auxiliary agents including, but not limited to, diluents, stabilizers (i. e., sugars and amino acids), preservatives, wetting agents, emulsifying agents, pH buffering agents, viscosity enhancing additives, colors and the like.

Further agents can be added to the composition of the invention. For instance, the composition of the invention may also comprise other anti-fungal agents well known in the art.

The amount of compounds obtained by the method of the invention is preferably a therapeutically effective amount. A therapeutically effective amount of compound obtained by the screening/selecting method of the invention is the amount necessary to allow the same to perform their biofilm formation inhibitor role without causing overly negative effects in the host to which the composition is administered. The exact amount of compounds obtained by the method of the invention to be used and the composition to be administered will vary according to factors such as the mode of administration, as well as the other ingredients in the composition.

The composition of the invention may be given to an animal through various routes of administration. For instance, the composition may be administered in the form of sterile injectable preparations, such as sterile injectable aqueous or oleaginous suspensions. These suspensions may be formulated according to techniques known in the art using suitable dispersing or

wetting agents and suspending agents. The sterile injectable preparations may also be sterile injectable solutions or suspensions in non-toxic parenterally-acceptable diluents or solvents. They may be given parenterally, for example intravenously, intramuscularly or sub-cutaneously by injection, by infusion or *per* os. Suitable dosages will vary, depending upon factors such as the amount of each of the components in the composition, the desired effect (short or long term), the route of administration, the age and the weight of the mammal to be treated. Any other methods well known in the art may be used for administering the composition of the invention.

Yet, another embodiment of the invention is to provide a method for preventing and/or treating candidiasis in an animal, the method comprising the step of administering to the animal a therapeutically effective amount of a composition as defined above. Preferably, the animal is a human, and even more preferably an immunocompromised human. An "immunocompromised subject" is an individual whose immune system is compromised or not functioning in a normal manner. For example, AIDS patients and patients undergoing immunotherapy are immunocompromised subjects. Further, various other conditions, such as certain cancers, viral infections and autoimmune diseases may render the affected individuals immunocompromised. Individuals who have undergone stem cell replacement therapy, bone marrow transplant, chemotherapy, radiotherapy and the like are also typically immunocompromised subjects.

The present invention further provides kits for use within any of the above detection and diagnostic methods. As described herein after, such kits typically comprise two or more components necessary for performing a detection or diagnostic assay. Components may be compounds, reagents, containers and/or equipment.

Accordingly, there is provided a kit for the detection of the presence or absence of a *Candida* biofilm, comprising:

- at least one antibody that specifically binds to a *Candida* Yak1, Epa7 or Epa6 protein;
- a reagent to detect polypeptide-antibody immune complex;
- optionally a biological reference sample lacking Yak1, Epa7 or Epa6 polypeptides; and
- optionally a comparison sample comprising Yak1, Epa7 or Epa6 polypeptides which specifically bind to said antibody;

wherein said antibody, reagent, biological reference sample, and comparison sample are present in an amount sufficient to perform said detection.

In a first preferred embodiment of the detection kit of the invention, the *Candida* strain is *Candida glabrata* and the at least one antibody specifically recognises the Yak1, Epa7 and/or Epa6 polypeptide. In a second preferred embodiment of the detection kit of the invention, the *Candida* strain is *Candida albicans* and the at least one antibody specifically recognises the Yak1 polypeptide.

There is also provided a kit for the early diagnostic of a *Candida* strain forming a biofilm, comprising:

- specific primers for *Candida* EPA6, EPA7 and/or YAK1 genes;
- specific probes for *Candida* EPA6, EPA7 and/or YAK1 genes;
- reagents for detecting the mRNA level of EPA6, EPA7 and/or YAK1 genes;
- a control sample of *Candida* cell culture at an early planktonic growth phase;
- optionally a biological reference sample lacking EPA6, EPA7 and/or YAK1 polynucleotides; and

- optionally a comparison sample comprising EPA6, EPA7 and/or YAK1 polynucleotides which specifically hybridize with said primers or probes;

wherein said primers, probes, reagents, biological reference sample, and comparison sample are present in an amount sufficient to perform said determination.

Specific primers for Candida EPA6 and EPA7 genes used in accordance with the diagnostic kit of the invention are preferably the following :

For EPA6 gene :

RT-EPA6F 5'cgctgtttgatacattaccac 3' (SEQ ID NO: 12)

RT- EPA6-7R 5' gaaggagtactattggtgatcg 3' (SEQ ID NO: 13).

For EPA7 gene :

RT-EPA7F 5' ccgaattagatcatttaccgg 3' (SEQ ID NO: 14)

RT- EPA6-7R 5' gaaggagtactattggtgatcg 3' (SEQ ID NO: 13).

As used herein, "specific probes" for Candida EPA6, EPA7 or YAK1 gene encompass polynucleotides substantially identical to the nucleic acid sequences of Candida EPA6, EPA7 or YAK1 polynucleotides as mentioned above or fragments thereof that retain the capacity to specifically hybridize with Candida EPA6, EPA7 or YAK1 polynucleotides as mentioned above under stringent conditions commonly used by the skill in the art.

The present invention will be more readily understood by referring to the following examples. These examples are illustrative of the wide range of applicability of the present invention and is not intended to limit its scope. Modifications and variations can be made therein without departing from the spirit and scope of the invention. Although any methods and materials similar or equivalent to those described herein can be used in the practice for testing of

the present invention, preferred methods and materials are described hereinafter.

EXAMPLES

5

EXAMPLE 1

The yak1p kinase controls expression of adhesins and biofilm formation in *Candida glabrata* in a sir4p dependent pathway

10 Biofilm is the predominant type of microbial development in natural environments, and potentially represents a major form of resistance or source of recurrence during host infection. Although a large number of studies have focussed on the genetics of bacterial biofilm formation, very little is known about the genes involved in this type of growth in fungi. A genetic screen for *Candida*
15 *glabrata* Biofilm mutants was performed using a 96-well plate model of biofilm formation. This study of the isolated mutant strains allowed the identification of four genes involved in biofilm formation (*RIF1*, *SIR4*, *EPA6* and *YAK1*). Epa6p is a newly identified adhesin required for biofilm formation in this pathogenic yeast. *EPA6* and its close paralogue *EPA7* are located in sub-telomeric regions
20 and their transcription is regulated by Sir4p and Rif1p, two proteins involved in sub-telomeric silencing. Biofilm growth conditions induce the transcription of *EPA6* and *EPA7*: this is dependent on the presence of an intact sub-telomeric silencing machinery and is independent of the Mpk1p signalling pathway. Finally, the kinase Yak1p is required for expression of both adhesin genes and
25 acts through a sub-telomeric silencing machinery-dependent pathway.

The aim of this study was to identify *C. glabrata* genes involved in biofilm formation. To date, two different approaches have been used to study biofilm genetics. On one hand, the use of insertional mutant collections has been particularly effective in identifying genes required for the early steps in bacterial
30 biofilm formation (O'Toole *et al.*, 1999). On the other hand, global expression analysis comparing cells growing under biofilm conditions to those in a

planktonic state of growth has revealed new elements which are specifically regulated during biofilm maturation (Whiteley *et al.*, 2001; Schembri *et al.*, 2003; Beloin *et al.*, 2004). Similarly, transcriptome analysis has confirmed the unique physiology of *C. albicans* cells during biofilm formation (García-Sánchez *et al.*,
5 2004).

In this study, a genetic screening strategy was used to identify genes involved in biofilm formation in *C. glabrata*. The inventors identified a new protein (Epa6p) which is the principal adhesin involved in biofilm formation in this yeast. Finally, the inventors demonstrated that the expression of *EPA6* is
10 regulated both by the Yak1p kinase and a biofilm signal which involve a pathway dependent on the sub-telomeric silencing machinery.

Results

15 *C. glabrata* strain BG2 forms biofilm

When incubated in a 24 well plate and in the presence of a plastic slide, *C. glabrata* grew as a thin biofilm on this plastic surface where *C. glabrata* formed tridimensional structures (Figure 1). Although the technique used to prepare the cells does not usually allow the observation of the exopolymeric
20 matrix, residual polymeric structures are visible at the highest magnification. In contrast with *C. albicans* biofilms, usually composed of a mix of yeast and hyphae, *C. glabrata* biofilms are composed of budding yeasts only with no evidence of filamentation or pseudo filamentation on any media tested in this study.

25 When *C. glabrata* strain BG2 was incubated in a microtiter plate, similar biofilm growth was observed on the surface of the wells. Less than 0.1% of the cells in the wells were in the planktonic state. Moreover, no cell accumulation at the bottom of the wells due to sedimentation was visible. After washing out the less adherent cells, the number of cells strongly adherent to the plastic surface
30 was estimated using a colorimetric assay based on XTT reduction (Ramage *et al.*, 2001). By analyzing the relationship between the cells growth phase and their ability to adhere to the plastic surface and form biofilm, we showed that

C. glabrata cells in the stationary phase had greater adherence than those in the logarithmic growth phase (Figure 2A).

Identification of C. glabrata mutant strains with altered ability to form biofilms

5 An insertional mutant library (Cormack and Falkow, 1999) was used to isolate *C. glabrata* strains with increased ability to form biofilm (phenotype Biofilm++) or those which were partially or completely defective in terms of biofilm formation (phenotype Biofilm-). The inventors plated 5000 mutant cells on YPD, replicated them individually into 96-well microtiter plates and incubated
10 the resulting cultures at 37°C (see Experimental procedures). After elimination of the least adherent cells, we used the XTT assay to estimate the quantity of cells which adhered strongly to the plastic surface. So as to be able to identify Biofilm++ and Biofilm- strains, measurements were made after 1 hour of incubation with XTT - this is a time point at which XTT is not completely reduced
15 by the wild type strain (Figure 2B). the inventors identified 72 Biofilm- mutants (chosen arbitrarily as having less than 30% of the number of adherent cells seen with the wild type strain) and 44 Biofilm++ mutants (chosen arbitrarily as having more than 200% of the number of adherent cells seen with the wild type strain).

20 The eight mutants that yielded the lowest XTT reductase activity and the twelve that yielded the highest activity were selected for further analysis. The inventors confirmed that these Biofilm mutant strains had the same growth rate under planktonic conditions as the wild type strain (with the same media and temperature used to grow biofilms). Moreover, as the enzyme reducing XTT in
25 yeast is a mitochondrial dehydrogenase (Baillie and Douglas, 1999), the inventors checked that these Biofilm mutant strains were not affected in their mitochondrial metabolism by plating them on YPG (2% glycerol) medium. Figure 3A shows a typical example of the structures produced by Biofilm- and Biofilm++ strains in the thermanox slide model of biofilm formation. In synthetic
30 medium, the original strain and the Biofilm- strain (CG129) produce small structures that differ by their size and density. In the other hand, the Biofilm++ strain (CG137) formed large tridimensional structures that nearly completely

covered the surface of the plastic slide. In the 96-well plate model of biofilm formation, and after washing away the less adherent cells from the well surface, the original BG2 strain showed small colonies of between two cells and one hundred cells adhering to the plastic, whereas Biofilm- mutants were seen to be
5 single cells or as colonies of less than ten cells (Figure 3B). However, the total number of cell clusters on the plastic surface did not seem to be affected in these two mutants, as compared to the wild type strain. In contrast, a confluent or near-confluent layer of cells was observed for Biofilm++ mutant strains.

Of the 20 mutants studied, the inventors were able to identify 15 insertion
10 sites for the plasmid used for insertional mutagenesis. For the five remaining strains, the inventors were unable to recover any plasmid: it is likely that these plasmids had undergone too many alterations and that the insertion site could not be sequenced. The sequences obtained were compared by blast analysis, with the genome sequence of *C. glabrata* strain ATCC2001 (Dujon *et al.*, 2004).
15 A high proportion of insertion sites were intergenic (7 out of 15). This result is in good agreement with a previous analysis of the mutant library used in this study, which showed that insertion sites for many of these strains were located in intergenic regions (Cormack and Falkow, 1999). The present example will focus on a detailed analysis of four mutant strains (two Biofilm++ and two
20 Biofilm-).

Epa6p is a newly identified adhesin involved in biofilm formation

In the CG122 strain (Biofilm-), the *URA3*-cassette was inserted 1019 bp upstream of a 2145-nucleotide open reading frame (CDS1045.1) encoding a
25 protein 35% identical to the previously identified Epa1p adhesin (Cormack *et al.*, 1999). This newly identified *EPA* gene was designated *EPA6*. Epa6p shares all the features of an Epa protein, i.e. a peptide signal, a C-terminal putative transmembrane domain, numerous putative N- glycosylation sites and a serine-/threonine-rich domain of 295 amino acids respectively (Frieman *et al.*, 2002). In
30 the ATCC2001 strain, the *EPA6* gene is located within one of the two subtelomeric regions of chromosome 3. A 10 kb sequence 98% identical to the *EPA6* DNA sequence was identified at the other sub-telomeric end of this

chromosome (Dujon *et al.*, 2004). The inventors reasoned that this *EPA6* paralogue could be present in the BG2 genome as well. The inventors therefore amplified the complete *EPA6* ORF from BG2: restriction analysis of the amplified PCR product indeed revealed the presence of another *EPA6*-related
5 gene, which was named *EPA7* (Figure 4A). These two *EPA* genes encode proteins of 715 and 714 amino acids respectively and share 95% percent identity at the nucleotide level. We deleted *EPA6* by replacing a large part of the open reading frame by the *URA3* marker (see Figures 4A and 4B). One should note that the disruption cassette used to delete *EPA6* could also theoretically
10 disrupt *EPA7*. However, none of the 60 screened transformants carried mutations in the *EPA7* gene, whereas 6 independent *epa6* *::URA3* strains were obtained.

The inventors next tested the respective contributions of the Epa adhesins to *C. glabrata* biofilm formation. Whereas the quintuple *epa1Δ-epa5Δ*
15 mutant strain was very slightly affected in terms of biofilm formation ($75 \pm 14\%$) as compared to the original strain (Figure 5A), the *epa6Δ* strains displayed a clear Biofilm- phenotype, with $30 \pm 5\%$ adherent cells as compared to the parent strain (Figure 5A). The characteristics of this biofilm defect match those observed in the originally isolated *epa6-1* mutant, indicating that Epa6p is the
20 main adhesin involved in biofilm formation in *C. glabrata*.

EPA6 and EPA7 expression is induced in biofilm

An *EPA6-EPA7* sequence comparison allowed the inventors to identify specific primers for each gene that could be used to study their respective
25 transcription under planktonic and biofilm culture conditions through RT-PCR analysis. The specificity of the *EPA6* primers was confirmed using genomic DNA from the *epa6Δ* strain, with genomic DNA from BG2 as a positive control. The results of the RT-PCR experiments are presented in Figure 5B and demonstrated an increase (2.4 ± 0.3 fold) in *EPA6* transcript levels in biofilm,
30 compared to planktonic cultures. The inventors did not observe PCR amplification products from RNAs of *epa6Δ* mutant strains, thereby confirming the specificity of the *EPA6* primers. The study of the expression of *EPA6* at

different stages of growth in planktonic condition (Figure 5C) demonstrated that *EPA6* gene is transcribed at the highest level during the late stationary growth phase, consistent with the increased ability of stationary phase cells to form a biofilm and the role of Epa6p in this process. This contrasts with other *EPA* genes previously identified, which are either expressed during exponential growth (*EPA1*) or not at all (*EPA2-EPA5*) (Cormack *et al.*, 1999; De las Penas *et al.*, 2003). Very low levels of the *EPA7* transcript were detected in planktonic cultures, while (as with *EPA6*) increased levels were observed in biofilm (Figure 5B). Similarly, *EPA1* to *EPA5* were not expressed in planktonic cultures. These results demonstrate that the expression of *EPA6* and *EPA7* is induced in biofilm, and show the existence of a response to the biofilm lifestyle.

EPA6 and EPA7 transcription is controlled by YAK1

Further analysis of the Biofilm- mutant strains revealed that in two cases the plasmid had inserted within an ORF encoding a protein with 58% similarity at the amino acid level to the *S. cerevisiae* Yak1p protein. Originally isolated as a suppressor of a *ras1* temperaturesensitive mutant, *YAK1* deletion conferred growth on a *tpk1 tpk2 tpk3* triple mutant strain in *S. cerevisiae* (Garret and Broach, 1989). Yak1p participates in a pathway parallel to that of the cAMP-dependent protein kinase (PKA): it has overlapping targets but antagonistic effects, and seems to be involved in mediating the starvation signal (Werner-Whashburne *et al.*, 1991). The inventors transformed the BG14 strain (Ura-) using an *EcoRI* digested plasmid containing the *URA3* gene and the sequences surrounding the insertion site from the original *yak1* mutant strain: 90% of the transformants displayed the same Biofilm- phenotype as the original mutant. As assayed by XTT analysis, the transformants had $30 \pm 18\%$ adherent cells, compared to the wild type strain (Figure 5A). As the numbers of adherent cells for the *yak1* (CG129) and the *epa6Δ* (CG164) mutant strains were nearly identical (see Figure 2), the inventors studied transcription levels for *EPA6* and *EPA7* in the *yak1* mutant strains. As shown in Figure 5B, *YAK1* is required for the transcription of *EPA6* and *EPA7* under both planktonic and biofilm conditions.

EPA6 transcription is regulated by sub-telomeric silencing

When the inventors studied the growth of the *epa6Δ* strain (CG164) on SD and FOA media, they noticed that it was able to grow on both media (Figure 6). This result indicated that some of the *epa6Δ* cells expressed the *URA3* gene (allowing strains to grow on SD) whereas the remainder had a silent *URA3* gene (allowing them to grow on FOA). In *S. cerevisiae*, epigenetic gene silencing has been shown to occur at the silent mating-type loci *HML* and *HMR* or within sub-telomeric regions and the tandem rDNA array (Sherman and Pillus, 1997; Lustig, 1998). At subtelomeric loci, silencing is mediated by a multiprotein complex within which the Sir2/3/4 silent information regulator proteins play critical roles (Huang, 2002). The telomeres contain multiple Rap1p-binding sites that recruit the Sir complex: the Sir-Rap1 interaction is then competed by two Rap1p interacting factors (Rif1p and Rif2p) (Huang, 2002). In the ATCC2001 strain, *EPA6* is located in a sub-telomeric region of chromosome 3. Moreover, in two Biofilm++ mutants, insertion sites were located just upstream of genes encoding proteins involved in sub-telomeric silencing. Thus, the CG137 and CG140 strains had the plasmid insertion site 925 bp and 430 bp upstream of the *SIR4* homologue (CDS0129.1) and *RIF1* homologue (CDS0069.1) of *C. glabrata*, respectively. CgSir4p and CgRif1p show 17.8% and 24.8% amino acid identity with their respective *S. cerevisiae* orthologues. When *SIR4* was deleted in an *epa6 ::URA3* mutant strain, the variegation of *URA3* gene expression was abolished and the *epa6Δ sir4Δ* double mutant strain (CG170) was not able to grow on FOA medium (Figure 6). As expected, *sir4Δ* (CG160) and *rif1Δ* (CG149) strains displayed a Biofilm++ phenotype, thus confirming that sub-telomeric silencing regulates biofilm formation in *C. glabrata* (Figure 7A). Accordingly, *rap1-21* and *sir3* strains (De las Penas *et al.*, 2003) were also Biofilm++ (Figure 7A).

Measurements of *EPA6* expression in these strains showed that the Biofilm++ phenotype was associated with significant overexpression of the *EPA6* gene (Figure 7B). However, the *epa6Δ sir4Δ* double mutant strain (CG170) displayed the same level of biofilm formation as the single *sir4Δ*

mutant strains: this shows that in a *sir4Δ* background, the *EPA6* gene is not necessary for the expression of a Biofilm++ phenotype (Figure 7B). One should note that in the *sir4Δ* and to a lower extent in the *rif1Δ* strains, *EPA7* and *EPA1-EPA5* were also overexpressed suggesting that other Epa proteins could be subject to sub-telomeric silencing in this context (Figure 7B).

The biofilm signal and Yak1p act through a sub-telomeric silencing -dependent Mpk1p independent pathway

In order to determine whether or not Yak1p regulation of *EPA6* transcription was dependent on the sub-telomeric silencing machinery, the inventors constructed a *yak1 sir4Δ* double mutant strain. As shown in Figure 8A, a *yak1* mutation in a *sir4Δ* context did not affect cell adherence, and *yak1 sir4Δ* strains were Biofilm++. Moreover, *EPA6* and *EPA7* transcription levels were still higher than in the original strain. These results show that Yak1p regulates the transcription of *C. glabrata* adhesins via a sub-telomeric silencing-dependent mechanism.

In order to determine whether the biofilm signal responsible for the increase of *EPA6* expression in biofilm as compared to planktonic growth conditions was acting via regulation of sub-telomeric silencing, the inventors compared *EPA6* and *EPA7* expression in a *sir4Δ* strain under both circumstances. As shown in Figure 8B, no apparent further increase of the *EPA6* and *EPA7* transcription levels were detectable in biofilms (compared to planktonic growth conditions) in any of the sub-telomeric silencing mutant strains. Thus, a mutation in any of the proteins involved in sub-telomeric silencing seems to suppress the inducibility of *C. glabrata* adhesin encoding genes in response to biofilm growth conditions. This shows that the biofilm signal regulates sub-telomeric silencing.

In *S. cerevisiae*, regulation of sub-telomeric silencing by stress (but not by nitrogen starvation) acts via Sir3p phosphorylation by the Mpk1p kinase (Ai *et al.*, 2002), i.e. part of the cell wall integrity pathway. We reasoned that Mpk1p could be also a key kinase for the regulation of sub-telomeric silencing in response to biofilm growth conditions. We identified the *MPK1* gene in the *C.*

glabrata genome. It encodes a protein showing 76 % identity with the *S. cerevisiae* homologous protein. Even though the deletion of Mpk1p increased *EPA6* and *EPA7* transcript levels during planktonic growth, it did not abolish its biofilm dependent inducibility (Figure 8). The biofilm signalling pathway therefore appears to be Mpk1p independent.

Discussion

EPA6 encodes the main adhesin for biofilm formation in C. glabrata

In this study, the inventors demonstrated that Epa6p represents the main adhesin involved in biofilm formation in *C. glabrata*. Indeed, *epa6Δ* strains are Biofilm-, and *EPA6* transcription is induced by biofilm growth conditions. Epa7p does not seem to play a major role in biofilm formation, since *yak1* strains which do not express *EPA6* and *EPA7* had a Biofilm phenotype comparable to that of *epa6Δ* strains. However, *epa6Δ* strains do not seem to be impaired in terms of adherence to a plastic surface *per se*. As shown in Figure 3B, it is the size of the colonies adhering to the plastic surface that is affected in *epa6Δ* strains, not their number. This result suggests that Epa6p is not involved in adherence to the plastic surface as such but rather in cell-cell adherence within the biofilm. The fact that the closest homologue of Epa proteins in *S. cerevisiae* is Flo11p (a protein known to be involved in cell flocculation and in biofilm formation) supports this hypothesis (Reynolds and Fink, 2001; Halme *et al.*, 2004).

Theoretically, the adhesin candidate responsible for adherence to a plastic surface should be expressed under planktonic and biofilm growth conditions but should not be subject to Yak1p regulation since *YAK1* deletion does not prevent adherence to the plastic (see below). In a recent report, De la Penas and colleagues estimated that they may be at least 16 different *EPA* genes in the *C. glabrata* ATCC2001 genome (De las Penas *et al.*, 2003). It is not yet known whether one or more of the other Epa proteins or other types of adhesin (i.e. glucan- or protein-based) are responsible for adherence to plastic in *C. glabrata*.

Yak1p acts through a sub-telomeric silencing machinery dependent pathway in C. glabrata

The inventors demonstrate here that Yak1p is necessary for *EPA6* and *EPA7* expression. Moreover, these phenotypes are bypassed by a mutation in one of the genes encoding proteins necessary for sub-telomeric silencing. These results show that Yak1p acts through a sub-telomeric silencing-dependent mechanism. It remains to be found whether Yak1p is itself regulated by biofilm growth conditions. In *S. cerevisiae*, Yak1p has been shown to be regulated at three levels: firstly, its specific activity is regulated by nitrogen starvation and stationary phase status (Garret *et al.*, 1991). Secondly, the same signals also regulate the level of expression of its encoding gene. Thirdly and finally, nuclear localization is induced by glucose starvation or rapamycin treatment (Schmelzle *et al.*, 2004; Moriya *et al.*, 2001). In response to glucose removal, Yak1p associates with Bmh1p-Bmh2p, enters the nucleus and phosphorylates Pop2p, a component of a multiprotein complex that regulates gene expression both positively and negatively (Moriya *et al.*, 2001). Yak1p regulates also the nucleocytoplasmic localization of the PKA regulatory subunit Bcy1p (Griffioen *et al.*, 2001). The action of Yak1p thus appears to be very complex and multifactorial. Furthermore, in *Dictyostelium discoideum*, YakA is necessary for starvation-induced growth arrest and the starvation-induced induction of genes necessary for development (Mendes Souza *et al.*, 1998). In *C. glabrata*, *YAK1* transcription level was not affected by biofilm growth conditions, although this kinase seems to be necessary for shifting from planktonic growth to growth under biofilm conditions.

The present working model for the regulation of sub-telomeric silencing by biofilm growth in *Candida glabrata* is presented in Figure 9. In this model, expression of the *Epa* adhesins is controlled by subtelomeric silencing which in turn is regulated by a biofilm signal acting through or independently of Yak1p.

Modulation of sub-telomeric silencing by Rif1p and Mpk1p in C. glabrata

The results presented here show that *C. glabrata* adhesins are regulated by a biofilm signal and Yak1p via a sub-telomeric silencing machinery-

dependent mechanism. In yeast, Sir3p is the limiting factor for propagation of silencing in sub-telomeric regions (Stone and Pillus, 1998) and the only kinase identified to date as being involved in Sir3p phosphorylation (and subsequently in the regulation of sub-telomeric silencing) is Mpk1p (Ai *et al.*, 2002). In response to stress (but not to nitrogen starvation), Mpk1p is activated and phosphorylates Sir3p, thus repressing sub-telomeric silencing. This leads to expression of the *PAU* genes located in the sub-telomeric region encoding cell wall proteins (Ai *et al.*, 2002). Additional factors (such as starvation, heat shock or the presence of mating pheromone) can affect Sir3p phosphorylation and sub-telomeric silencing (Stone and Pillus, 1996; Ai *et al.*, 2002). In this study, the inventors demonstrated that biofilm-induced regulation of sub-telomeric silencing was not dependent on Mpk1p. However, the function of Mpk1p in *C. glabrata* seems to differ slightly from that of its *S. cerevisiae* counterpart. Indeed, deletion of *MPK1* in *C. glabrata* increases *EPA6* transcription, most probably due to modulation of sub-telomeric silencing, whereas in *S. cerevisiae* deletion of the same gene does not modify the constitutive level of sub-telomeric silencing (Ai *et al.*, 2002). The function of Rif1p seems also to be different in *C. glabrata* when compared to *S. cerevisiae*. Indeed, in the latter organism, Rif1p acts as a negative regulator of telomeric silencing and length (Moretti *et al.*, 1994), whereas deletion of *RIF1* in *C. glabrata* derepresses the expression of the *EPA* genes located within sub-telomeric regions. These results suggest that the overall mechanisms of sub-telomeric silencing are not conserved between these two hemiascomycetous yeasts, even though the same constitutive elements are present.

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Variegation of EPA6 expression suggests phenotypic heterogeneity of the cell surface in C. glabrata

The inventors demonstrated that in a *epa6Δ::URA3* cell population, some cells were Ura⁺ and others were Ura⁻. This result shows that in a BG2 cell population, some cells express the *EPA6* gene at the cell surface whilst others do not. Similar results were obtained with the other *EPA* genes identified to date (De las Penas *et al.*, 2003). Moreover, previous studies in *S. cerevisiae* have

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shown that the degree of silencing within sub-telomeric regions differed from one chromosome end to another (Pryde and Louis, 1999). One can thus imagine that the Epa organization at the cell surface of a clonal *C. glabrata* cell population is highly heterogeneous.

5

Experimental procedures

Strains and media

Candida glabrata strains used in this study are listed in Table 1: all strains were isogenic to the BG2 strain (Fidel *et al.*, 1996). The SC and –Ura DO synthetic media and the YPD rich medium were prepared as previously described (Sherman, 1992). When needed, 5-fluoroorotic acid (FOA) was added to SC medium at 1g/l. The bacterial strain *Escherichia coli* XL1-blue (Stratagene, La Jolla, CA) was used for propagation of all plasmids. All procedures for manipulating DNA were performed as previously described (Sambrook *et al.*, 1989).

15

Biofilm formation

C. glabrata strains were grown to stationary phase with agitation in SC medium at 37°C. Cells were then harvested by centrifugation, washed in sterile water and resuspended in SC or YPD medium at a cell density adjusted to OD600 = 1. For the plastic slide model of biofilm formation, these cell suspensions were distributed into prestelirized polystyrene 24-well plates (TPP) in which a round plastic slide was added (Thermanox, Nunc). After 24h of growth at 37°C, the culture medium was eliminated firstly by inverting the plates and secondly by careful pipetting. Biofilm were then examined by scanning electronic microscopy (SEM) as previously described (Prigent-Combaret *et al.*, 2000). Briefly, biofilm samples were fixed for 1 h in 0.07 M sodium cacodylate buffer (pH 7.3) containing 1.2% glutaraldehyde and 0.05% ruthenium red. The samples were then washed in the same buffer containing 0.05% ruthenium red and postfixed in 1% osmium tetroxide in cacodylate buffer, treated by the critical point drying method, and observed on a Gemini DSM 982 scanning electron microscope.

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Transmission and scanning electron microscopy were performed by Brigitte Arbeille and Claude Lebos at the Laboratoire de Biologie Cellulaire et Microscopie Electronique, UFR Médecine, Tours, France. For the microtiter plate model of biofilm formation, the cell suspension in SC medium were distributed (100 µl per well) into presterilized, polystyrene 96-well microtiter plates (TPP, round bottom) and incubated overnight at 37°C. Following biofilm formation, less adherent cells were removed by washing the plates three times in water and once in PBS buffer. Strongly adherent cells were quantified by using the previously described metabolic 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT, Sigma) reduction assay (Ramage *et al.*, 2001). Briefly, XTT solution was prepared at 0.5 mg/ml in PBS, filter-sterilized and stored at -20°C. Prior to each assay, an aliquot of stock XTT was thawed and methadione (1 µM final) was added. A 100 µl aliquot of the XTT-methadione solution was added to each well. The microtiter plates were then incubated for 1 hour at 37°C. A colorimetric change was measured at 492 nm using a microtiter plate reader (Labsystems Multiskan RS).

Characterization of the URA3-cassette insertion site in the Biofilm mutant strains

The mutant library used for screening for Biofilm mutant strains has been previously described (Cormack *et al.*, 1999). In order to determine the sequence of the *URA3*-cassette insertion site, the genomic DNA was extracted (Philippson *et al.*, 1991) and digested by *EcoRI*. After ligation and amplification in *E. coli*, a plasmid containing the *URA3*-cassette and the genomic flanking regions was recovered. The sequence of the insertion site was then determined using M13-40 and M13-REV48 as sequencing primers.

Gene disruption

The disruption cassette was constructed by PCR fusion using a similar strategy to that used by Kuwayama and colleagues (Kuwayama *et al.*, 2002). For *EPA6*, upstream and downstream gene fragments and the *URA3* marker were PCR-amplified using the HFPCR kit from Clontech (Palo Alto, CA). The primers used

for these amplifications are listed in Table A (supplementary material), and their positions are shown in Figure 4. EPA6-3'5 and EPA6-5'3 contain the reverse complements of MKRfEPA6 and MKRrEPA6, respectively. In addition, 5 ng of each of the three gel-purified, amplified fragments were used as substrates for PCR fusion with the primers EPA6-5'5 and EPA6-3'3 and the following programme: 94°C for 30s, followed by 35 cycles of 94°C for 15s and 68°C for 4 min. The final PCR fragment represented the *epa6* ::*URA3* cassette. The BG14 (*ura3*) strain was transformed with the deletion cassette as previously described (Cormack and Falkow, 1999), and Ura⁺ transformants were then selected on – URA DO plates. Proper integration of the cassette was determined by PCR using a primer that annealed to a region outside the disruption cassette (EPA6ex) and a primer that annealed to a sequence within the marker (*URA3F*) (Figure 4A, Table A). The inventors screened 55 colonies and identified 6 putative, homologous integrants. A second pair of primers was used to check for correct integration of the cassette (EPA6ex2-*URA3R*) (Figure 4A, Table A). PCR amplifications of the EPA6ex – EPA6ex2 region were used to verify the knock-out of the wild-type gene in each putative deletion strain (Figure 4A, Table A). Furthermore, Southern blot analysis was used to confirm gene deletion. A similar strategy was used to disrupt *SIR4*, *RIF1* and *MPK1*. Concerning *YAK1*, the plasmid recovered from the original *yak1*::*URA3* strains was used to transform the strain BG14 after *EcoRI* restriction. Primers for both outer regions of the cassette in the *C. glabrata* genome were used to screen for transformants having integrated the cassette in the *YAK1* locus. Southern blot analysis was performed to confirm gene deletion.

So as to construct a double mutant *sir4Δ epa6Δ* and *sir4Δ yak1* strain, a spontaneous Ura⁻ derivative of a *sir4Δ*::*URA3* strain was isolated on FOA medium. The stability of the Ura⁻ phenotype was tested by growing the cells on YPD medium and plating them on FOA and SD media. The inventors also checked that the Biofilm++ phenotype was not affected by this mutation. This *sir4Δ*::*ura3* strain was then used as a recipient strain for constructing the *sir4Δ epa6Δ* and *sir4Δ yak1* double mutants, using the same strategy as with the corresponding single mutant strains.

RNA preparation and RT-PCR

Cells were grown to stationary phase on SC medium, under agitation for planktonic growth conditions or in microtiter plates for biofilm growth conditions.

5 Total RNA from 10^6 cells was prepared as previously described (Schmitt *et al.*, 1990). The purified RNA was treated with DNase to eliminate any trace of DNA, and was then used to isolate the mRNA using the oligotex mRNA kit (Qiagen). RT-PCR was performed by using the Access RT-PCR system kit (Promega) and an Icyler (Biorad) thermocycler. After RNA denaturation (68°C for 5min),
10 the reverse transcription step was performed at 48°C for 45 min followed by 2min at 94°C. PCR amplifications were carried out as follows: 2 min at 94°C (one cycle) 94°C for 30 sec, 58°C for 1 min, 68°C for 1 min (30 cycles) and 68°C for 7 min (one cycle). The primers used for the RT-PCR are listed in Table A (supplementary material). *ACT1* was used as the control. For each
15 preparation and each couple of primers, PCRs without the reverse transcription step were performed to check that preparations were free of DNA. Three independent RNA preparations were prepared from each strain and growth condition. In order to compare the expression of *EPA6* in each strain and each growth condition, serial dilutions of the PCR products (*ACT1* and *EPA6*)
20 obtained with the BG2 strain under planktonic growth conditions were deposited in an agarose gel, and curves of the ratio of the fluorescence of the bands and the dilutions were drawn. The fluorescence of each electrophoretic band was measured using a VersaDoc Imaging System (Biorad) apparatus and Quantity One 4.3.1 software (Biorad). For each sample, appropriate dilutions of the PCR
25 products were deposited on agarose gels and their fluorescence was compared to the standard curves.

EXAMPLE 2

The mechanism of action of YAK1 on biofilm formation in C. albicans is different from the one previously identified in C. glabrata

5 YAK1 is necessary for biofilm formation in *Candida glabrata*. A strain in which YAK1 has been deleted produced only 30% of biofilm as compared to the original strain. We identified the target of YAK1 regulation in *C. glabrata*. Thus, the gene EPA6 which encodes an adhesin necessary for biofilm formation is not expressed in a *yak1Δ* strain. EPA6 as all the other members of this adhesins family identified to date is located within a sub-telomeric region of the *C. glabrata* genome and its transcription is regulated by the sub-telomeric silencing machinery. The present results show that YAK1 regulates the expression of EPA6 and consequently biofilm formation through the regulation of sub-telomeric silencing level.

15 In *Candida albicans*, none adhesin has been shown to be individually necessary for biofilm formation. This development state in *C. albicans* seems to be under the control of multiple factors. Moreover, no sub-telomeric localization of the *C. albicans* adhesin genes has been demonstrated. YAK1 in *C. albicans*, seems to act on biofilm formation through a mechanism completely different from the one we have identified in *C. glabrata*.

 Inactivation of the *CaYAK1* gene was conducted in *C. albicans* strain RM1000 (*ura3Δ::λimm434/ ura3Δ::λimm434 his1Δ::hisG/ his1Δ::hisG*; Alonso-Monge *et al.*, 2003) according to the procedures of Enloe *et al.* (2000) and Gola *et al* (2003). Briefly the *URA3* and *HIS1* markers of pFA-Ura and pFA-His, respectively (Gola *et al.*, 2003), were amplified using the following oligonucleotides and standard conditions for amplification :

30 Yak1-5p
5'-
CAAACAAAACGCAAGAGATCACATACCATTAATAATATATAAGACCAACCAT
TGTAACCACACAAAGTATCACAGTATCACCGACAATTTATACATAGAAGCT
TCGTACGCTGCAGGTC-3'

Yak1-3p
 3'-
 TAAACAAAATTAATTAATAAGTATCATTAAAGAGTATAAACTTAATACTGGT
 CAACCTCCCCCTCCCTCCACATTGTTATTCTTATTCTTATTCTTCTGATATCA
 5 TCGATGAATTCGAG

with the underlined regions corresponding to the pFA plasmids and the non-underlined regions corresponding to regions of the *caYAK1* gene (*YAK1* of *C. albicans*) located 5' or 3' of the open reading frame.

10 The resulting PCR products were used to transform *C. albicans* RIM1000 cells prepared according to Walther and Wendland (2003). Ura3+ or HIS1+ transformants were selected on appropriate media and the replacement of one copy of the *CaYAK1* gene in these transformants was confirmed by PCR using standard procedures. Ura3+ or His+ transformants were subsequently
 15 transformed by the PCR-amplified *HIS1* or *URA3* markers, respectively, using the same technique. Transformants were selected for prototrophy and replacement of the second *caYAK1* allele was confirmed by PCR.

The resulting *C. albicans ura3/ura3 his1/his1 yak1Δ::URA3/yak1Δ::HIS1* strains were tested for their ability to form biofilms in two model of biofilm
 20 formation on a plastic surface. The first model is based on the use of a micro-fermentor containing a Thermanox™ slide which has been previously described (Garcia-Sanchez *et al.*, 2004). In the second model, Thermanox™ slides are incubated at 37°C in minimal medium in a glass tube under continuous rotation for 2-48h. In both models the *C. albicans yak1Δ/yak1Δ* strains were unable to
 25 develop a biofilm of significant biomass. Yet, observation of the Thermanox™ slide obtained using the second biofilm model revealed that the mutant strain was able to form microcolonies on the plastic surface. These micro-colonies consisted mostly of yeast cells with some hyphal or pseudo-hyphal forms (figure 10).

30 The resulting *C. albicans ura3/ura3 his1/his1 yak1Δ::URA3/yak1Δ::HIS1* strains were tested for their ability to produce hyphae in Lee's medium (Lee *et al.*, 1975) which triggers the yeast-to-hypha transition in *C. albicans*. As shown in the figure 10, in contrast to wild-type *C. albicans* which readily forms hyphae

after a few hours of incubation in Lee's medium, no true hypha was visible with the mutant strain and only pseudo-hyphae were observed.

Taken all together, these data show that the Yak1 kinase is necessary for the switch from yeast-to-hypha growth and for the switch from planktonic to
5 biofilm growth in *C. albicans*.

EXAMPLE 3

Role of YAK1 on biofilm formation in C. albicans

10 Experimental procedures

Biofilm and cultures.

An inoculum was prepared from an early-stationary-phase culture grown in flasks at 30°C in an orbital shaker and diluted to an optical density at 600 nm
15 (OD₆₀₀) of 1. Biofilms were produced in microfermentors (Ghigo J.M., 2001). These consist of a glass vessel with a 40-ml incubation chamber where two glass tubes are inserted to drive the entry of medium and air. Used medium is evacuated through a third tube. Medium flow is controlled by a recirculation pump (Ismatec) and pushed by the pressured air. Plastic slides (Thermanox;
20 Nunc) glued to a glass spatula were immersed in the inoculum for 30 min at room temperature. After this adhesion period, the spatula was transferred to the chamber and incubated at 37°C, with the medium flow set to 0.6 ml/min and air supplied at 10⁵ Pa; under these conditions, the growth of the planktonic phase is minimized and most of the cells remain on the spatula. For biofilm formation *C.*
25 *albicans* strains were grown in 0.67% yeast nitrogen base (YNB; Difco) with 0.4 % glucose, uridine 80 mg/liter, arginine 100 mg/liter, histidine 100 mg/liter, methionine 200 mg/liter. After 40 hours, spatula was removed from microfermentors, biomass is scrapped from the thermanox, and then filtered on a 0, 22mm membrane, then dry at 70°C for 3 days. Amount of biomass is then
30 quantified by dry weight.

Germ tube formations.

Germ tubes formation was determined in Lee (Lee *et al.*, 1975) media at 37°C after 3 hours of induction, in the presence or absence of 4-Amino-1-tert-butyl-3-(1'-naphthylmethyl)pyrazolo[3,4-d]pyrimidine (NMMP1) (1mM). (Toronto Research Chemicals Inc. Canada) (Bishop *et al.*, 1999).

Engineered YAK1 protein kinase

Engineered of protein kinases that contain an unique binding pockets that are not present in any wild-type protein kinase have been described by Bishop *et al.*, 2000. Substitution of one amino acid in the ATP-binding sites conferred unique inhibitor-sensitivity to NMPP1, an ATP analogue. By sequence comparison with other protein kinases, we proposed that the conserved Phe at position 507 was part of the ATP-binding sites. A mutation of Phe (507) to Alanine was introduced using PCR techniques.

In this example, the inventors show that the Yak1 protein plays an important role in the formation of biofilm in *C. albicans*. Indeed, and as illustrated in figure 11, the inventors have restored the capacity of a double mutant $\Delta yak1/\Delta yak1$ of *C. albicans* to form biofilm by reintroducing in such a strain the wild-type gene of YAK1.

Secondly, the inventors have introduced an allele of the YAK1 gene sensitive to N-MPP1 in a double $\Delta yak1/\Delta yak1$ of *C. albicans* strain. As shown in figures 11, 12 and 13, in absence of N-MPP1, the mutated kinase is active and the double mutated strains containing the allele of the YAK1 gene sensitive to N-MPP1 have the capacity of forming normal filament when the filamentation is induced in LEE medium. Whereas and as shown in figures 12 and 13, in presence of low concentration of N-MPP1, the capacity of the mutated kinase is specifically inhibited and the strains have thus lost their capacity of forming filaments.

The inventors have further noted from assays with preformed filaments, that these filaments did not further develop but rather returned to their planktonic stage when the activity of the mutated kinase was inhibited by N-MPP1.

EXAMPLE 4

Identification of in vivo inhibitors of biofilm formation in Candida

5 The kinase focused collection (ChemDiv, Inc) will be screened to select inhibitors of biofilm formation in a Yak1p-dependent or -independent way. The 96-wells plate model of *C. glabrata* biofilm will be used in this procedure. In order to identify antibiofilm compounds acting through the inhibition of the Yak1 signalling pathway, two concomitant screens will be conducted. A first assay on
10 the wild-type strain will identify molecules able to inhibit biofilm formation. A second assay will be conducted using a *sir4* mutant strain since deletion of the *sir4* gene suppresses the biofilm-deficient phenotype of a *yak1* mutant strain. Molecules inhibiting biofilm formation through the inhibition of Yak1 should theoretically inhibit biofilm formation in a wild-type background but not in a *sir4*
15 background. Molecules identified through these assays will then be tested on *C. albicans*.

EXAMPLE 5

Identification of in vitro inhibitors of Yak1

20 Production of recombinant *C. albicans* Yak1 will be attempted using two different hosts (*Escherichia coli* and *Pichia pastoris*) and various constructs, depending on the type and position of the affinity tag fused to the Yak1
25 polypeptide. The *C. albicans* Yak1 is a large protein (809 aa) and contains, in its N-terminal part, 5 CTG codons which are coding for serine instead of leucine in this yeast. In order to avoid this problem, the N-terminal part of the gene will be synthesised *in vitro* using a codon usage optimal for the expression of the protein in *E. coli* and *P. pastoris*. The C-terminal part containing the kinase
30 active site will be directly PCR-amplified and the complete gene sequence will

be constructed. Culture protocols will be optimized and the best system will be selected for the production of the full-length polypeptide as a soluble, active protein. Purification will be carried out using the affinity tag (His6 or GST) fused to the polypeptide. The activity of the protein will be tested using a kinase assay
5 as previously used for the *S. cerevisiae* orthologue. The active full-length recombinant protein will be then used for the *in vitro* inhibitor screen.

A Fluorescent/Luminescent Kinase Assay adapted for screening in 96 well-plate format will first be set up. Following up, the activity of Yak1 will be tested in the presence of known serine/threonine kinase inhibitors. A collection
10 of 3000 molecules of the kinase focused library will be selected (ChemDiv, Inc) and tested for their inhibitory potency on the Yak1 activity. The results obtained on the assay on the recombinant protein will be compared to the *in vivo* tests on *C. albicans* and *C. glabrata*

Table 1. Strains used in this work.

<i>Strain name</i>	<i>Genotype</i>	<i>Reference</i>
BG2	Clinical isolate	(Fidel <i>et al.</i> , 1996)
BG14	<i>ura3Δ::Tn903Neo^R</i>	(Cormack and Falkow, 1999)
BG573	<i>URA3 (hyr1 epa1 epa2 epa3 epa4 epa5)Δ</i>	(De las Penas <i>et al.</i> , 2003).
BG592	<i>ura3Δ::Tn903 G418R rap1-21</i>	(De las Penas <i>et al.</i> , 2003).
BG676	<i>ura3Δ::Tn903 G418R sir3Δ</i>	(De las Penas <i>et al.</i> , 2003).
CG122	<i>epa6-1</i>	this work
CG129	<i>yak1::URA3</i>	this work
CG137	<i>sir4-1</i>	this work
CG140	<i>rif1-1</i>	this work
CG145	<i>sir4Δ::URA3</i>	this work
CG149	<i>rif1Δ::URA3</i>	this work
CG160	<i>sir4Δ::ura3</i>	this work
CG164	<i>epa6Δ::URA3</i>	this work
CG170	<i>sir4Δ::ura3 epa6Δ::URA3</i>	this work
CG172	<i>sir4Δ::ura3 yak1::URA3</i>	this work
CG174	<i>mpk1Δ::URA3</i>	this work

Table A. Sequence of primers used in this study.

Primer name	Primer sequence (5'→3')
SIR4-ex	TTTTTGGTCAAAGCTCACCCC
SIR4-5'5	TAGTACAGGCATAGTTGCACGTCG
SIR4-5'3	GGGTGTCGGGGCTGGCTTAACTAGAAATCCTGGGTTATGCTCGTG
MKRfSIR4	CACGAGCATAACCCAGGATTCTAGTTAAGCCAGCCCCGACACCC
MKRrSIR4	CGAGAGACAGTTTGGCTGGTGTAAAAAATAGGCGTATCACGAGGCC
SIR4-3'5	GGCCTCGTGATACGCCTATTTTTTACACCAGCCAAACTGTCTCTCG
SIR4-3'3	AAGTACCTACCGTATCCGTCCTGC
SIR4ex2	TCGACTCCAATGTTCTCTACCCC
RIF1ex	TTCAAAAAATGGTGAAGTACGCC
RIF1-5'5	CACGTTCAGTAAACGTCCAGTCTG
RIF1-5'3	GGGTGTCGGGGCTGGCTTAACTAGTGGGATGCAAATCAGGTCTG
MKRfRIF1	CAGACCTGATTTGCATCCCCTAGTTAAGCCAGCCCCGACACCC
MKRrRIF1	CCGGTTCACCTGGCAATAGAAGAAAAAATAGGCGTATCACGAGGCC
RIF1-3'5	GGCCTCGTGATACGCCTATTTTTTCTTCTATTGCCAGTGAACCGG
RIF1-3'3	TTTCTTCTGCAGATAACTGGCCC
RIF1ex2	AAAGCTCAATTCCTCAAATTCGCGC
EPA6ex	GAACTAACATTTCATGAACAGG
EPA6-5'5	TCAGGTCTAGAACACCCCTTGTTGG
EPA6-5'3	GGGTGTCGGGGCTGGCTTAACTCCAAGAGGAAATTCAGTAGGGTCC
MKRfEPA6	GGACCCTACTGAATTTCTCTTGGAGTTAAGCCAGCCCCGACACCC
MKRrEPA6	CCTGAAGCACTCCCCTGCTAGAAAAAATAGGCGTATCACGAGGCC
EPA6-3'5	GGCCTCGTGATACGCCTATTTTTTCTAGCAGTGGGAGTGCTTCAGG
EPA6-3'3	GGAACCCTGATCTCCATTAGCC
EPA6-ex2	CGCCAGGCATTATAATACCGC
MPK1ex	GGTTAGCAGAGTAATTGCTCGGC
MPK1-5'5	CTGACATAGCCAGCAGTGAGAG
MPK1-5'3	GTCATAGCTGTTTCTCTGACTGTAGTCTCCTCGCTTGCCCTC
MKRrMPK1	GAGGCAAGCGAGGAGACTACAGTCAGGAAACAGCTATGAC
MPK1-3'5	TACAACGTCGTGACTGGGACATTCTGGCATCACCTATGGG
MKRfMPK1	CCCATAGGTGATGCCAGAATGTCCCAGTCACGACGTTGTA
MPK1-3'3	GACCAGCAATGATGACACCTGA
MPK1ex2	TGGTTTCCTTGTATTGGAACCG
URA3F	TTTAGCGGCTTAACTGTGCCC
URA3R	GGAAGGGATGCTAAGGTAGAG
EPA6F	TTGTGAAGCGATTGAGCAGTGG
EPA6R	AGTGTGTGATAATCGTTAAGGC
RT-ACT1F	GGCTTTCGATTCTCACC
RT-ACT1R	GTGGCAACGGTTTGATGC
RT-EPA6F	CGCTGTTTGATACATTACCAC
RT-EPA7F	CCGAATTAGATCAATTTACCGG
RT-EPA6-7R	GAAGGAGTACTATTGGTGATCG

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Although preferred embodiments of the present invention have been described in detail herein and illustrated in the accompanying drawings, it is to
15 be understood that the invention is not limited to these precise embodiments and that various changes and modifications may be effected therein without departing from the scope or spirit of the present invention.

CLAIMS:

1. A method of screening for a compound that affects expression of a gene involved in biofilm formation of a *Candida* strain, said gene being selected from the group consisting of EPA6, EPA7 and YAK1, the method comprising the steps of:
 - providing a *Candida* biofilm with an initial expression level for said gene;
 - contacting said biofilm with at least one compound to be tested;
 - evaluating expression of the gene; and
 - identifying the compound that inhibits the initial expression level of said gene in the biofilm.
2. The method of claim 1, wherein the compound inhibits expression of gene EPA6, EPA7 and/or YAK1 by down-regulating the transcription of the gene EPA6, EPA7 and/or YAK1, or by down-regulating the translation of mRNA in a protein encoded by the gene EPA6, EPA7 and/or YAK1.
3. The method of claim 2, wherein the down-regulation of transcription is evaluated by measuring the mRNA level.
4. The method of claim 2, wherein the down-regulation of translation is evaluated by measuring the protein level.
5. The method of any one of claims 1 to 4, wherein the *Candida* strain consists of *Candida glabrata* or *Candida albicans*.
6. The method of any one of claims 1 to 5, wherein the *Candida* strain consists of *Candida glabrata* and the gene whose expression is evaluated is EPA6.

7. The method of any one of claims 1 to 5, wherein the *Candida* strain consists of *Candida glabrata* and the gene whose expression is evaluated is EPA7.
- 5 8. The method of any one of claims 1 to 5, wherein the *Candida* strain consists of *Candida glabrata* and the gene whose expression is evaluated is YAK1.
9. The method of any one of claims 1 to 5, wherein the *Candida* strain consists of *Candida albicans* and the gene whose expression is evaluated is YAK1.
- 10 10. The method of any one of claims 1 to 9, further comprising a step of evaluating inhibition of biofilm formation by the compound.
11. A compound that affects biofilm formation of a *Candida* strain obtained by the method as defined in any one of claims 1 to 10.
12. The compound as defined in claim 11, for use as an anti-fungal agent.
- 15 13. A composition for preventing and/or treating candidiasis, comprising a compound as defined in claims 11 or 12, and an acceptable carrier.
14. A method for an early diagnostic of a *Candida* strain forming a biofilm, comprising the steps of:
- a) obtaining a cell sample of said *Candida* strain;
- 20 b) measuring the mRNA level of EPA6, EPA7 and/or YAK1 genes in said cell sample; and
- c) measuring the mRNA level of said gene in a control *Candida* cell culture at early planktonic growth,
- wherein overexpression of EPA6, EPA7 and/or YAK1 genes in said cell sample compared to the control cell culture, is indicative of the presence of a *Candida* strain forming a biofilm.
- 25

15. The method of claim 14, wherein the mRNA level is quantified by RT-PCR.
16. The method of claim 14, wherein the Candida strain consists of *Candida glabrata* and the gene whose mRNA level is measured is EPA6, EPA7 and
5 YAK1.
17. The method of claim 14, wherein the Candida strain consists of *Candida albicans* and the gene whose mRNA level is measured is YAK1.
18. A method for detecting the presence or absence of a *Candida* biofilm in a sample, comprising the steps of:
- 10 a) contacting the sample with at least one molecule that specifically recognizes a Candida Yak1, Epa7 or Epa6 polypeptide for a time and under conditions sufficient to form a complex; and
- b) detecting the presence or absence of the complex formed in a).
19. The method of claim 18, wherein the molecule is an antibody that
15 specifically binds to said Yak1, Epa7 or Epa6 polypeptide, or functional derivative thereof.
20. The method of claim 18 or 19, wherein the Yak1 polypeptide comprises an amino acid sequence substantially identical to SEQ ID NO: 1 or functional derivative thereof.
- 20 21. The method of claim 20, wherein the functional derivative of said Yak1 polypeptide comprises an amino acid sequence substantially identical to SEQ ID NO:4.
22. The method of claim 18 or 19, wherein the Epa6 polypeptide comprises an amino acid sequence substantially identical to SEQ ID NO: 2.
- 25 23. The method of claim 18 or 19, wherein the Epa7 polypeptide comprises an amino acid sequence substantially identical to SEQ ID NO: 3.

24. The method of claim 18 or 19, wherein the *Candida* strain is *Candida glabrata* and the at least one molecule specifically recognises the Yak1, Epa7 and/or Epa6 polypeptide.
- 5 25. The method of claim 18 or 19, wherein the *Candida* strain is *Candida albicans* and the at least one molecule specifically recognises the Yak1 polypeptide.
26. A method for preventing and/or treating candidiasis in an animal, the method comprising the step of administering to the animal a therapeutically effective amount of a composition as defined in claim 13.
- 10 27. The method of claim 26, wherein said animal is a human.
28. The method of claim 26 or 27, wherein said human is an immunocompromised human.
29. Use of the compound of claim 11 in the preparation of a medicament for preventing or treating candidiasis.
- 15 30. Use of at least one *Candida* protein selected from the group consisting of Epa6, Epa7, Sir4, Rif1 and Yak1 or at least one gene encoding said protein as a target for identifying a compound capable of impairing biofilm formation of a *Candida* strain.
- 20 31. Use according to claim 30, wherein said *Candida* strain consists of *Candida glabrata* or *Candida albicans*.
32. Use according to claim 30, wherein the *Candida* protein consists of Epa6 protein of *Candida glabrata*.
33. Use according to claim 30, wherein the *Candida* protein consists of Epa7 protein of *Candida glabrata*.
- 25 34. Use according to claim 30, wherein the *Candida* protein consists of Sir4 protein of *Candida glabrata*.

35. Use according to claim 30, wherein the Candida protein consists of Rif1 protein of *Candida glabrata*.
36. Use according to claim 30, wherein the Candida protein consists of Yak1 protein of *Candida glabrata*.
- 5 37. Use according to claim 30, wherein the Candida protein consists of Yak1 protein of *Candida albicans*.
38. A kit for the detection of the presence or absence of a Candida biofilm, comprising:
- 10 - at least one antibody that specifically binds to a Candida Yak1, Epa7 or Epa6 protein;
- a reagent to detect polypeptide-antibody immune complex;
- optionally a biological reference sample lacking Yak1, Epa7 or Epa6 polypeptides; and
- 15 - optionally a comparison sample comprising Yak1, Epa7 or Epa6 polypeptides which specifically bind to said antibody;
- wherein said antibody, reagent, biological reference sample, and comparison sample are present in an amount sufficient to perform said detection.
39. The kit of claim 38, wherein the Candida strain is *Candida glabrata* and the at least one antibody specifically recognises the Yak1, Epa7 and/or Epa6 polypeptide.
- 20 40. The kit of claim 38, wherein the Candida strain is *Candida albicans* and the at least one antibody specifically recognises the Yak1 polypeptide.
41. A kit for the early diagnostic of a Candida strain forming a biofilm, comprising:
- 25 - specific primers for Candida EPA6, EPA7 and/or YAK1 genes;

- specific probes for *Candida* EPA6, EPA7 and/or YAK1 genes;
 - reagents for detecting the mRNA level of EPA6, EPA7 and/or YAK1 genes;
 - a control sample of *Candida* cell culture at an early planktonic growth phase;
 - optionally a biological reference sample lacking Epa6, Epa7 and/or Yak1 polynucleotides; and
 - optionally a comparison sample comprising EPA6, EPA7 and/or YAK1 polynucleotides which specifically hybridize with said primers or probes;
- 10 wherein said primers, probes, reagents, biological reference sample, and comparison sample are present in an amount sufficient to perform said determination.
42. A method for selecting a biofilm formation inhibitor of a *Candida* strain acting through a Yak1 dependent pathway, comprising the following steps:
- 15 a) contacting a compound with a biofilm of a wild type *Candida* strain and determining if it inhibits biofilm formation of said *Candida* strain;
 - b) contacting the compound selected in a) with a biofilm of a *sir4* mutant *Candida* strain and determining if it inhibits biofilm formation of said *Candida* strain;
 - 20 c) selecting the compound identified in a) if said compound inhibits the formation of biofilm of the wild-type *Candida* strain and does not inhibit the formation of biofilm of the *sir4* mutant *Candida* strain.
43. The method of claim 42, wherein said wild type and *sir4* mutant *Candida* strains consist of *Candida glabrata* strains.

44. The method of claim 43, wherein the *sir4* mutant *Candida* strain consist of *Candida glabrata* strain filed at the C.N.C.M. on November 30, 2005 under the reference number I-3532.

45. A compound obtained by the method of any one of the claim 42 to 44, said compound having the capacity of affecting biofilm formation of a *Candida* strain.

46. An *in vitro* screening method for an inhibitor of the activity of a Yak1 polypeptide of *Candida*, comprising the steps of :

a) contacting a purified or isolated Yak1 recombinant polypeptide or a fragment thereof retaining the kinase activity with a candidate compound;

b) measuring the capacity of said candidate compound to inhibit the kinase activity of the Yak1 polypeptide or fragment thereof, said kinase activity being measured by a fluorescent and/or luminescent kinase assay.

47. The method of claim 46, wherein the Yak1 recombinant polypeptide has an amino acid sequence substantially identical to SEQ ID NO: 1.

48. The method of claim 46, wherein the Yak1 recombinant polypeptide has an amino acid sequence substantially identical to SEQ ID NO: 4.

49. The method of claim 46, wherein the Yak1 recombinant polypeptide has an amino acid sequence substantially identical to SEQ ID NO: 10.

50. A compound obtained by the method of any one of claims 46 to 49, said compound having the capacity of affecting biofilm formation of a *Candida* strain

51. A method for selecting a biofilm formation inhibitor of a *Candida* strain acting through a Yak1 dependent pathway, comprising the implementation of the method of claim 42 and the method of claim 46 whatever the order the two methods are implemented.

52. A purified or isolated polypeptide involved in *Candida* biofilm formation comprising an amino acid sequence substantially identical to SEQ ID NO: 1.
53. A purified or isolated polynucleotide encoding the purified polypeptide of claim 52.
54. The polynucleotide of claim 53 comprising the nucleic acid sequence SEQ ID NO: 5.
55. A purified or isolated polypeptide having a kinase activity sensitive to N-MPP1 comprising the amino acid sequence SEQ ID NO: 9.
56. A *sir4* mutant *Candida* strain filed at the C.N.C.M. on November 30, 2005 under the reference number I-3532.

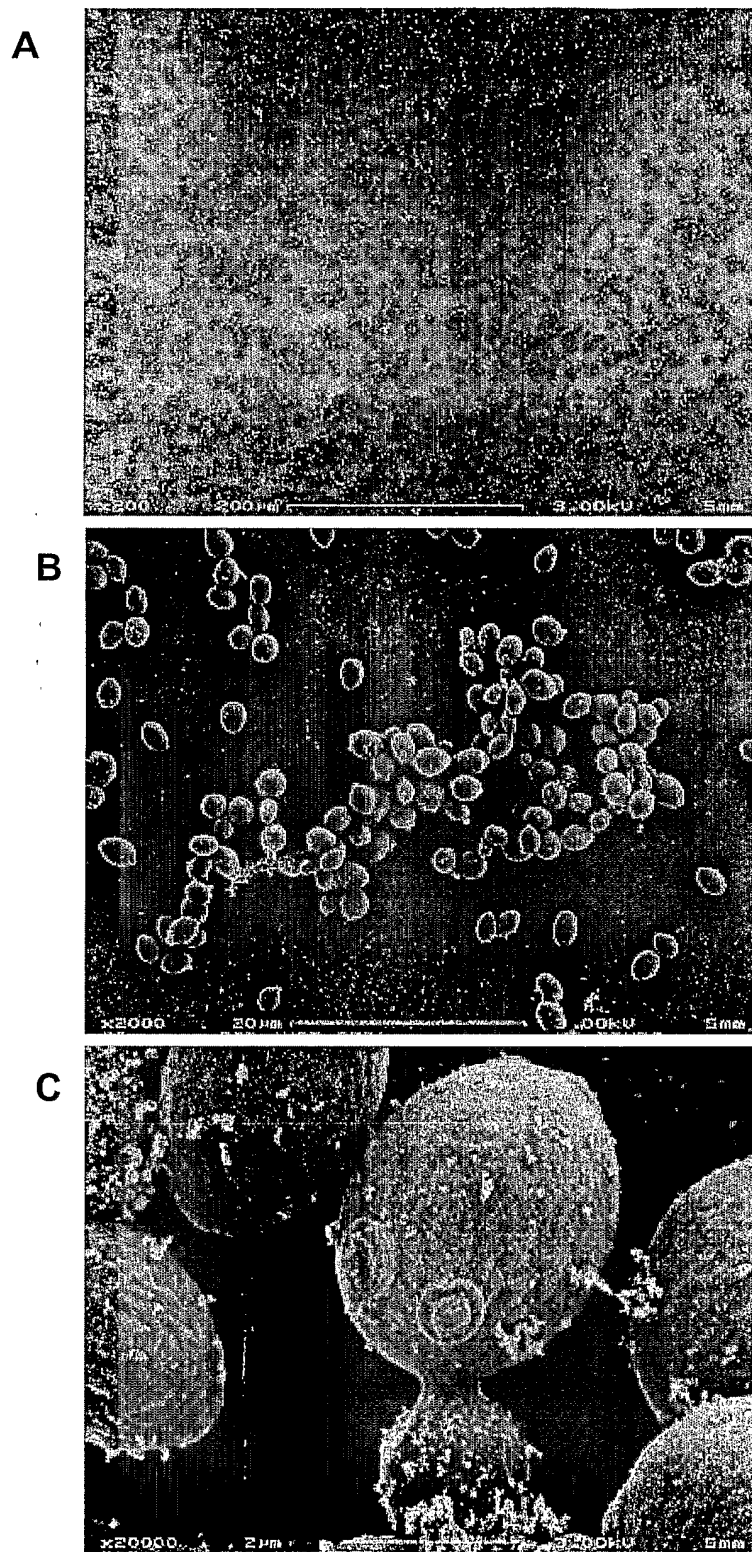
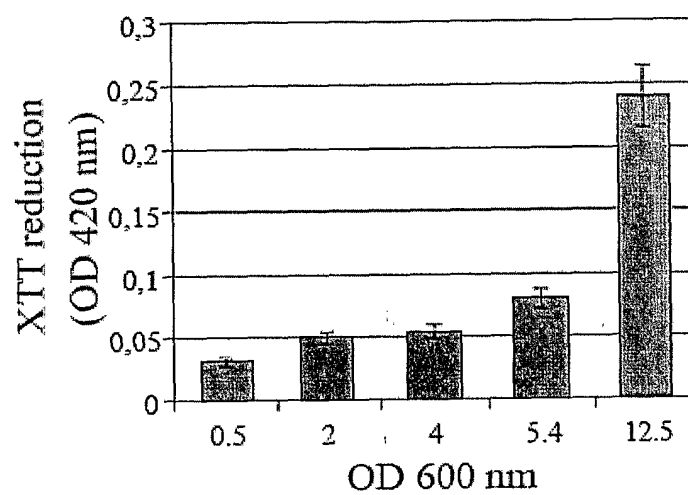
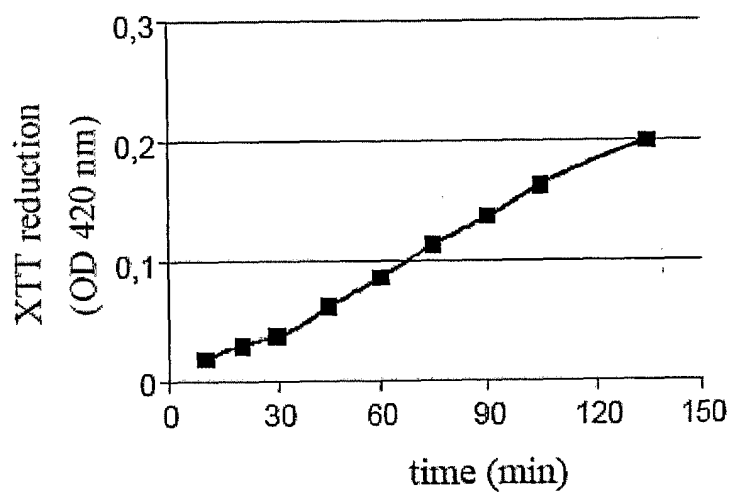


FIGURE 1

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A**B****FIGURE 2**

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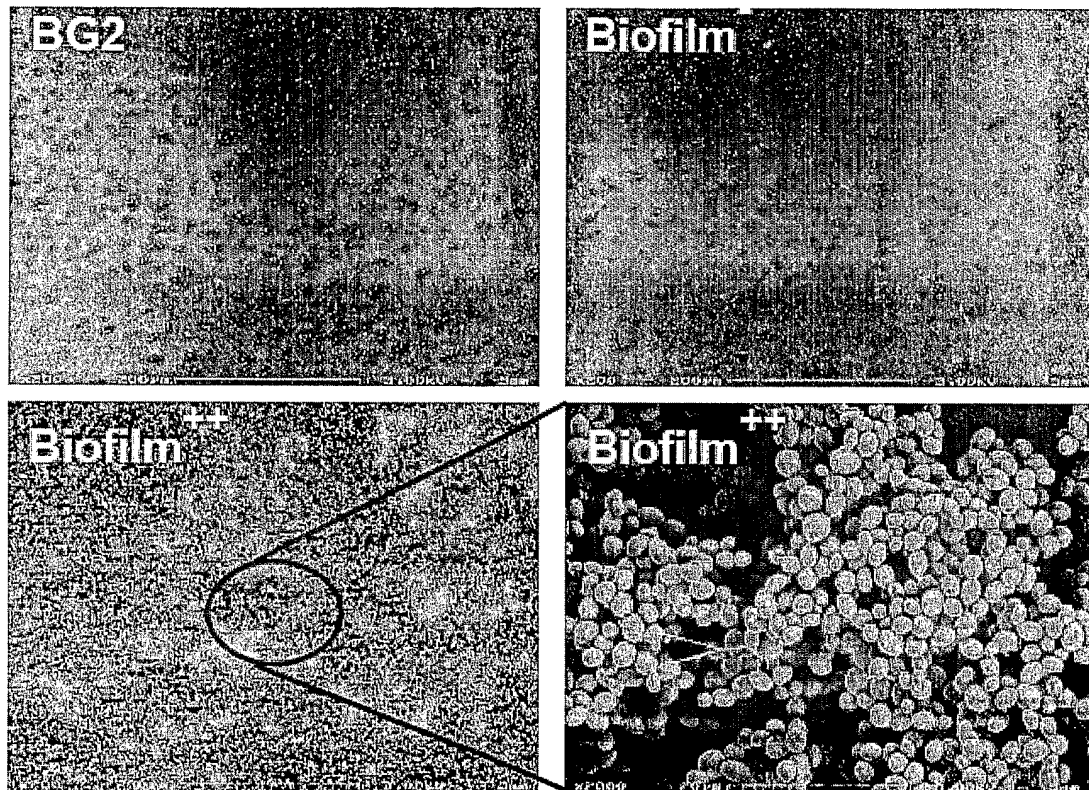


FIGURE 3A

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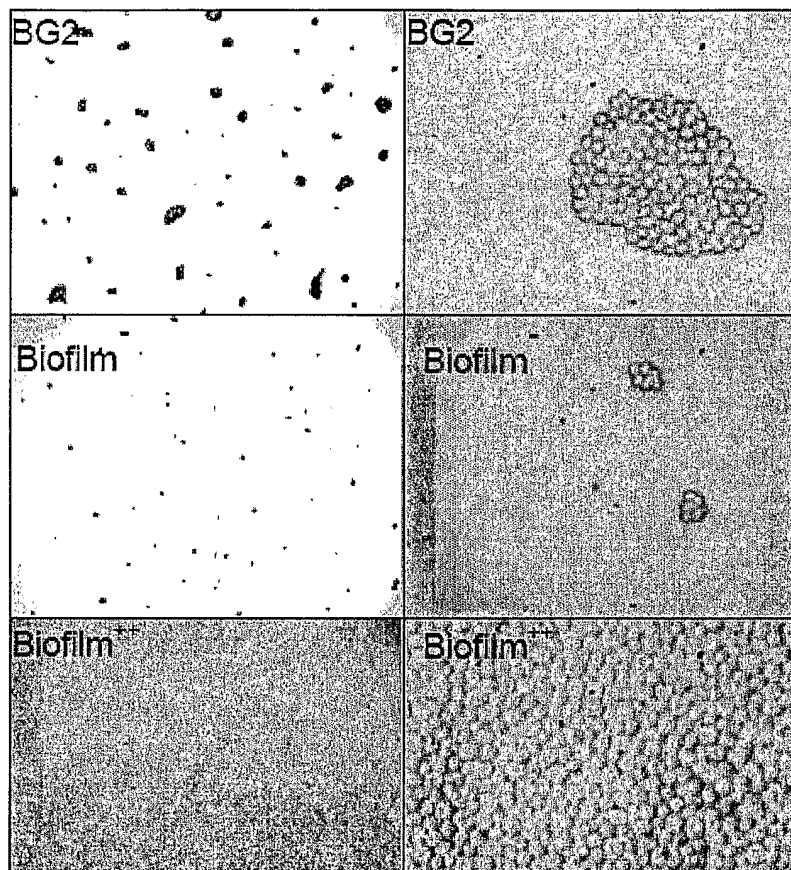


FIGURE 3B

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probe

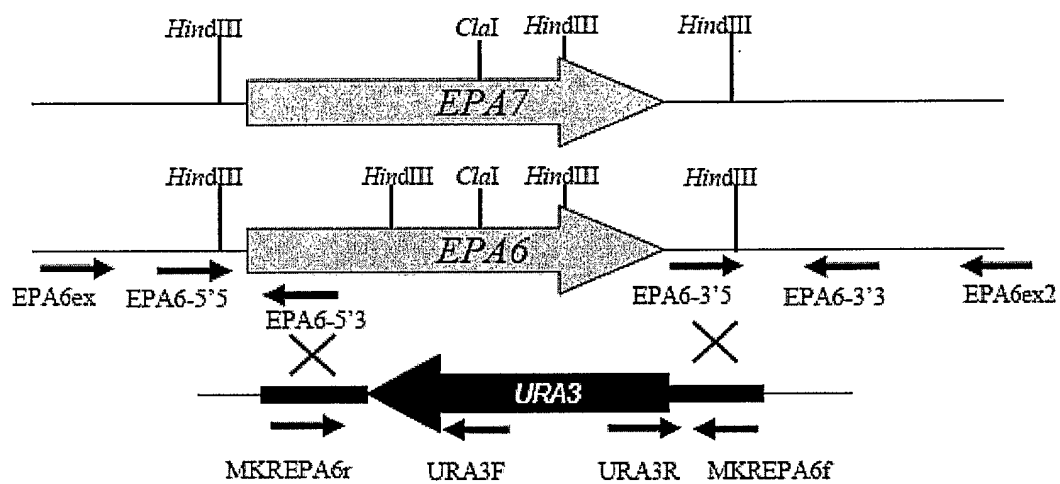


FIGURE 4A

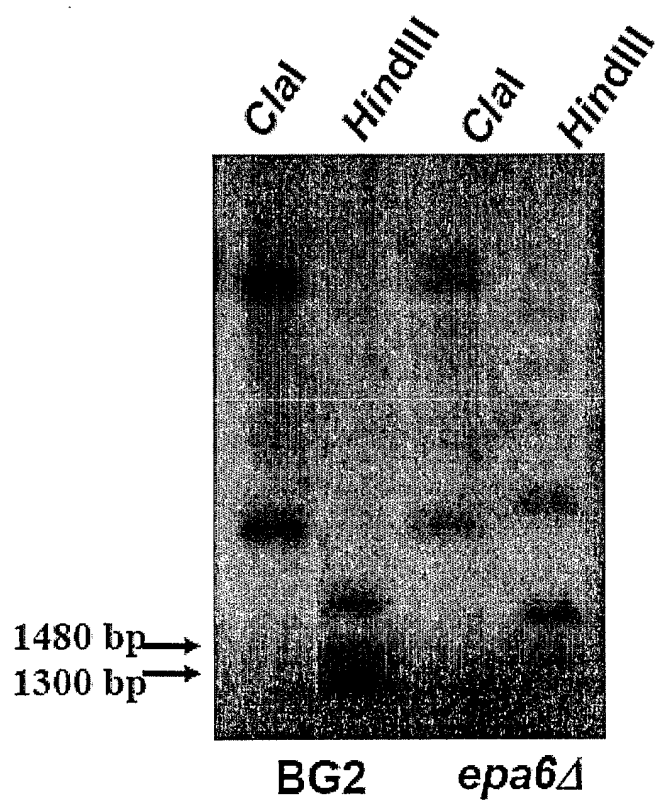
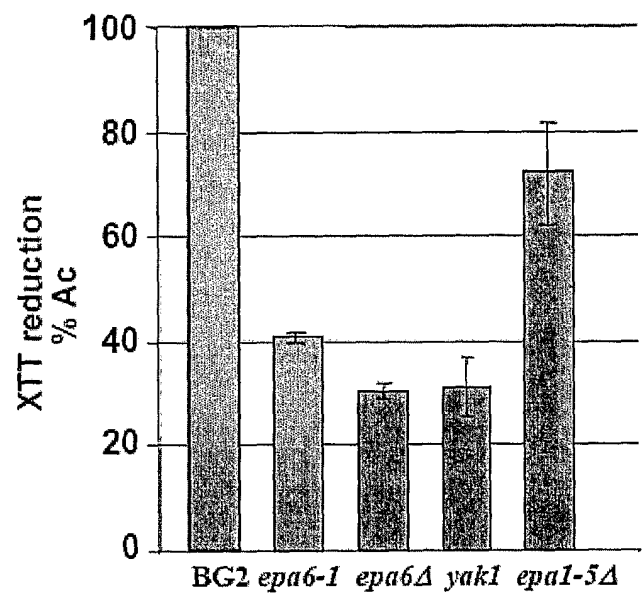


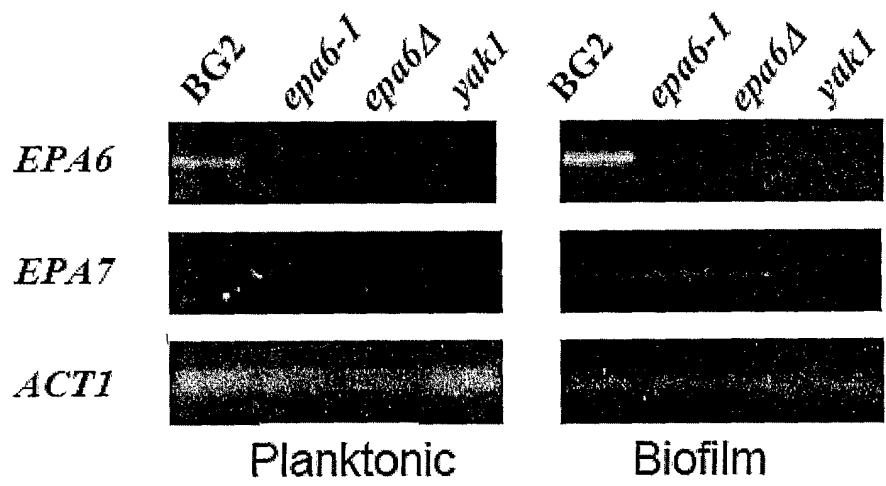
FIGURE 4B

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A



B



C

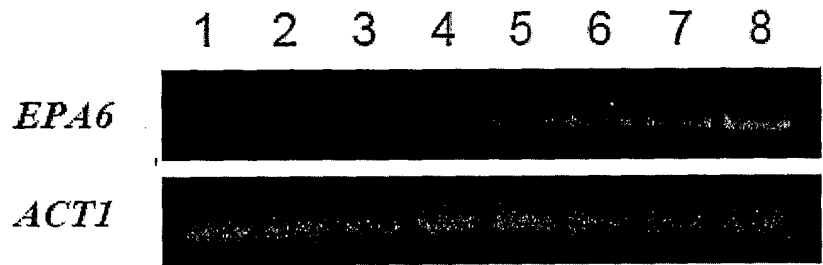


FIGURE 5

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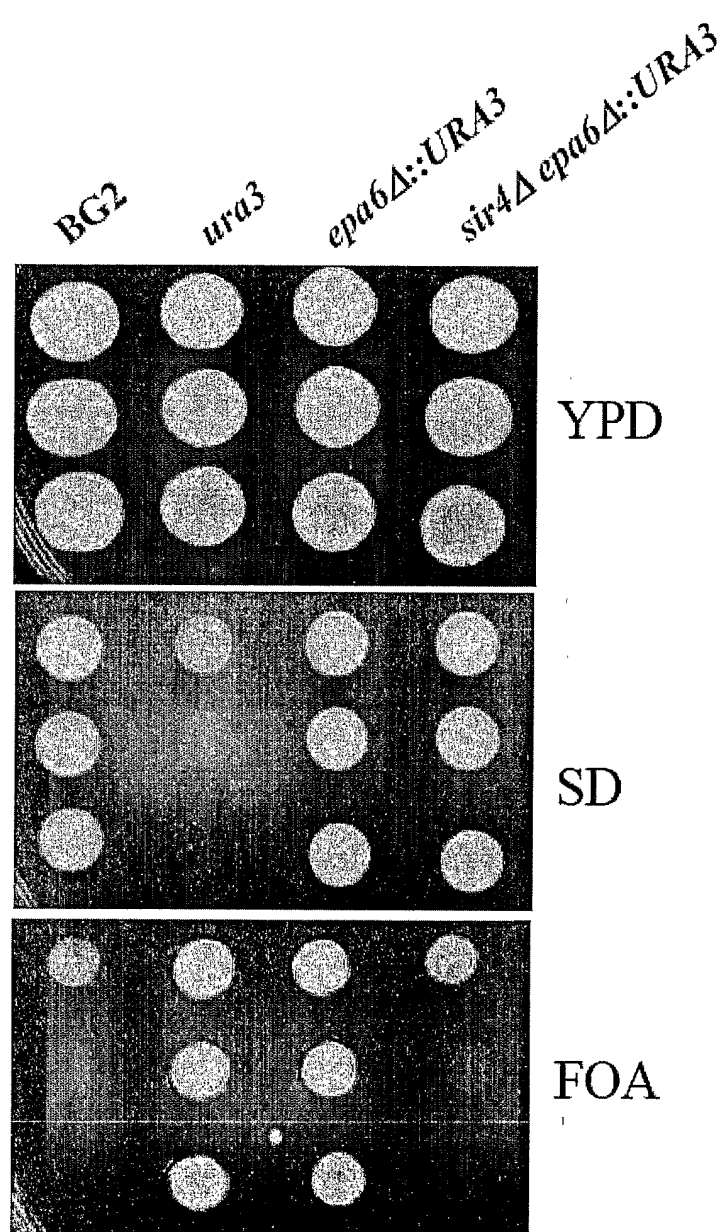


FIGURE 6

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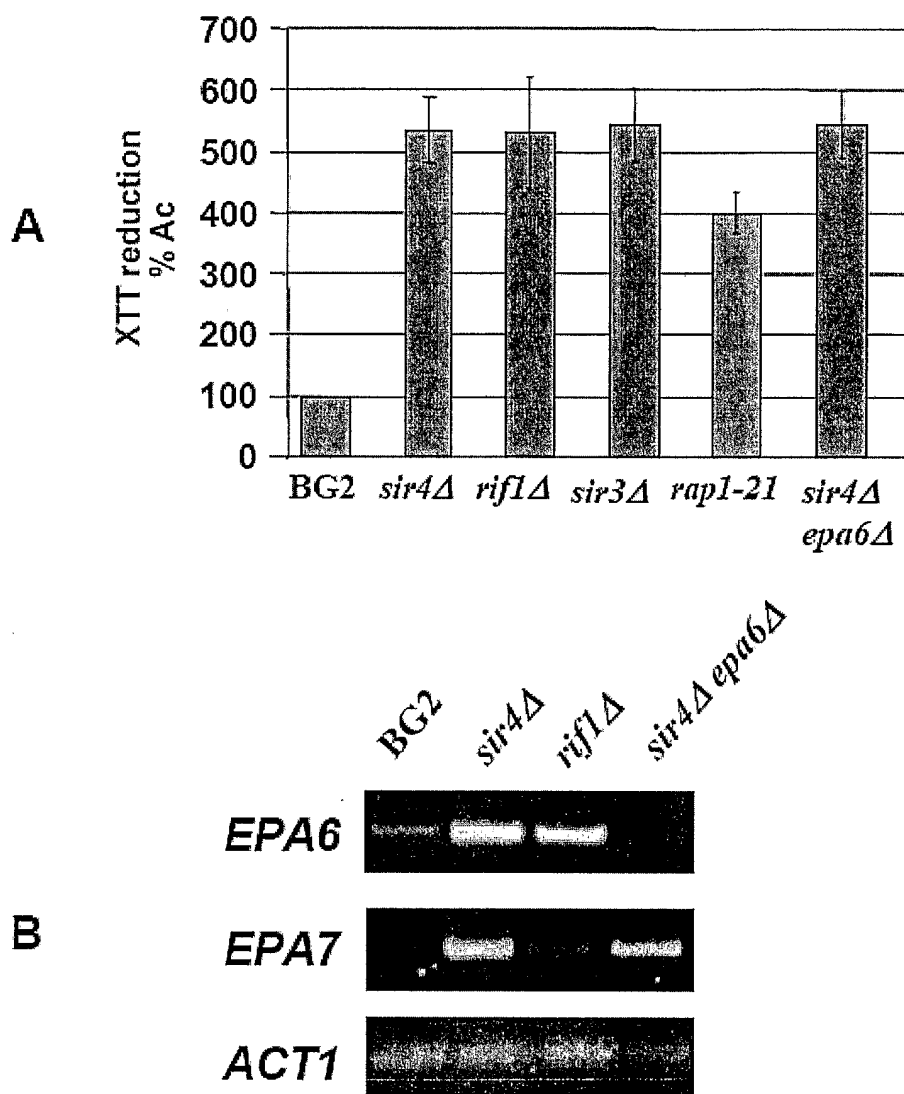


FIGURE 7

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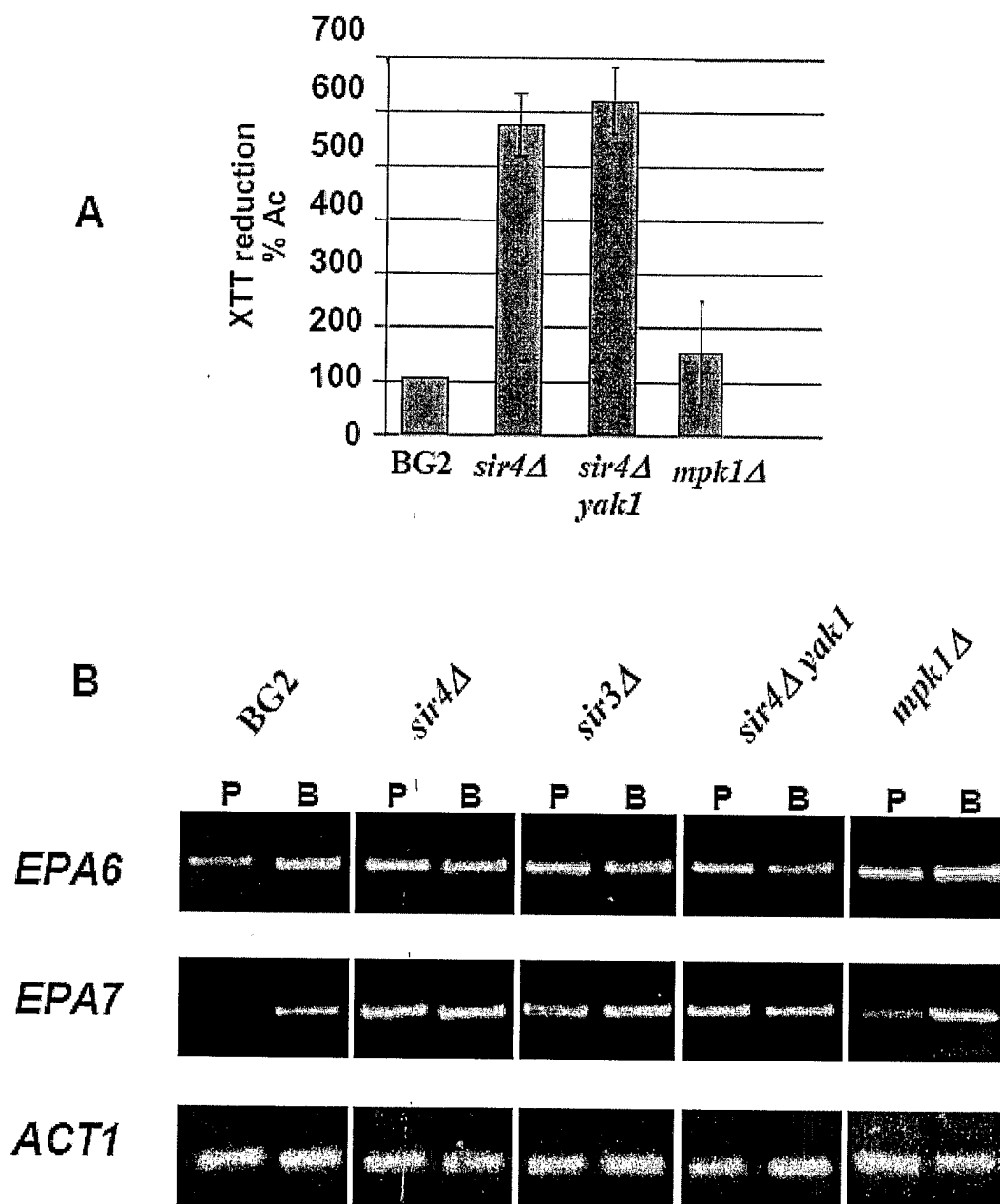


FIGURE 8

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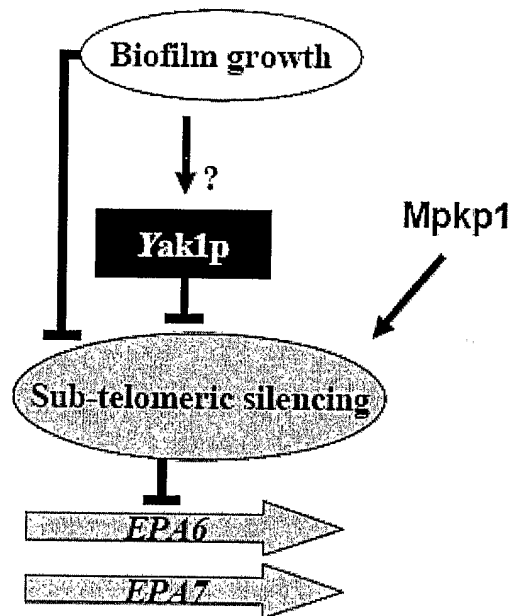


FIGURE 9

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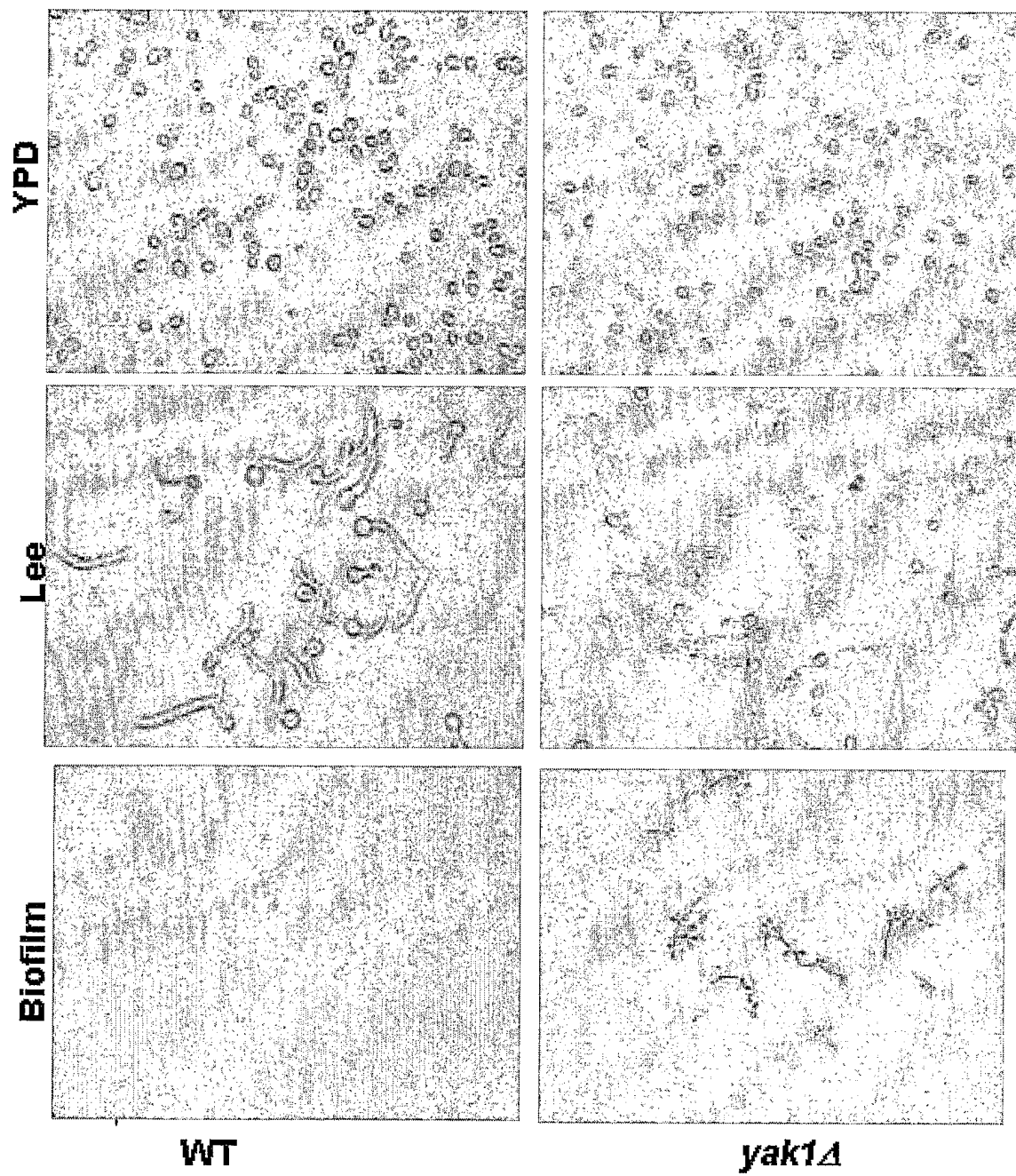


FIGURE 10

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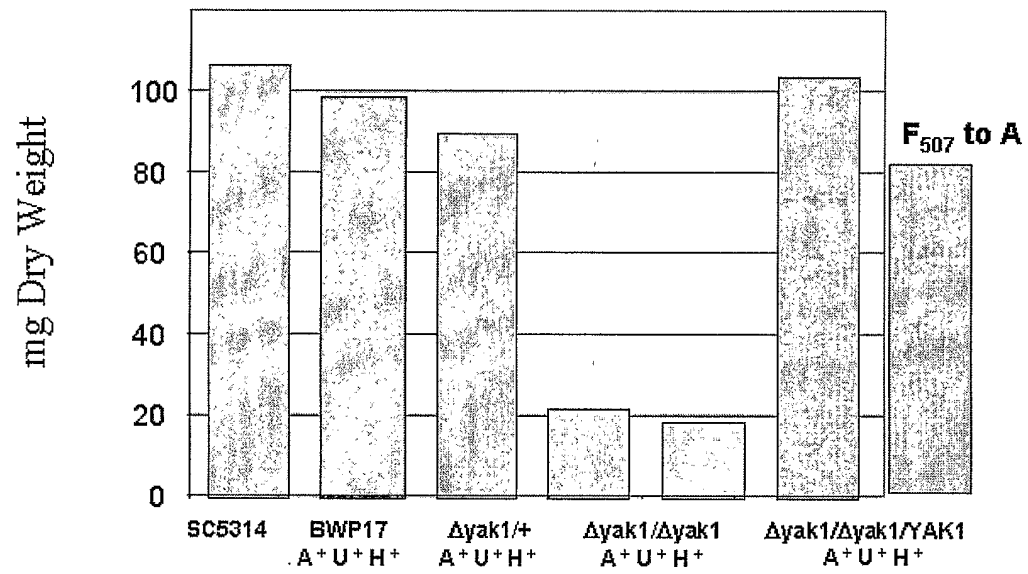


FIGURE 11

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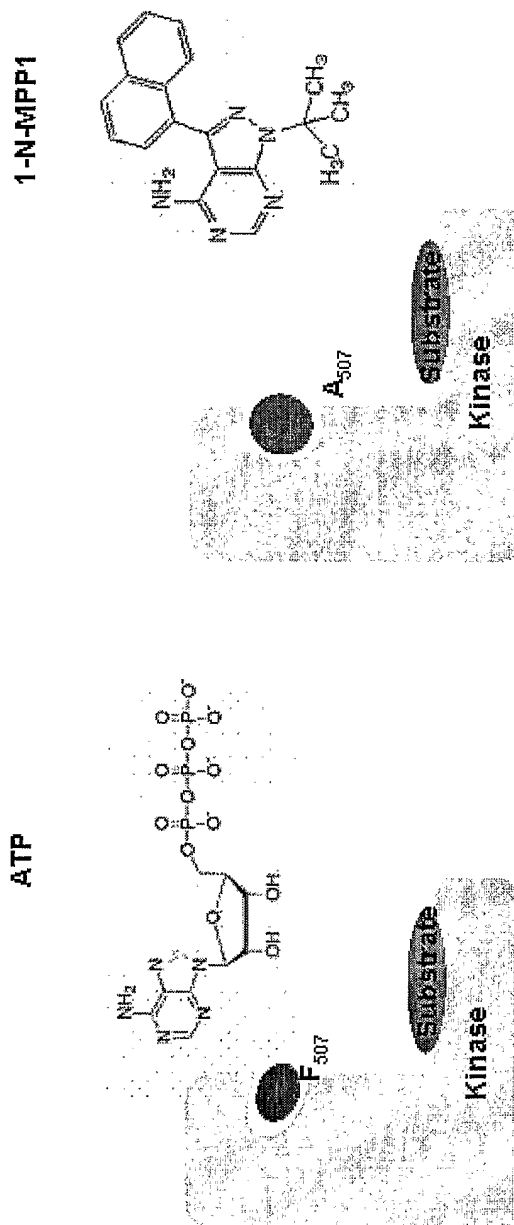


FIGURE 12

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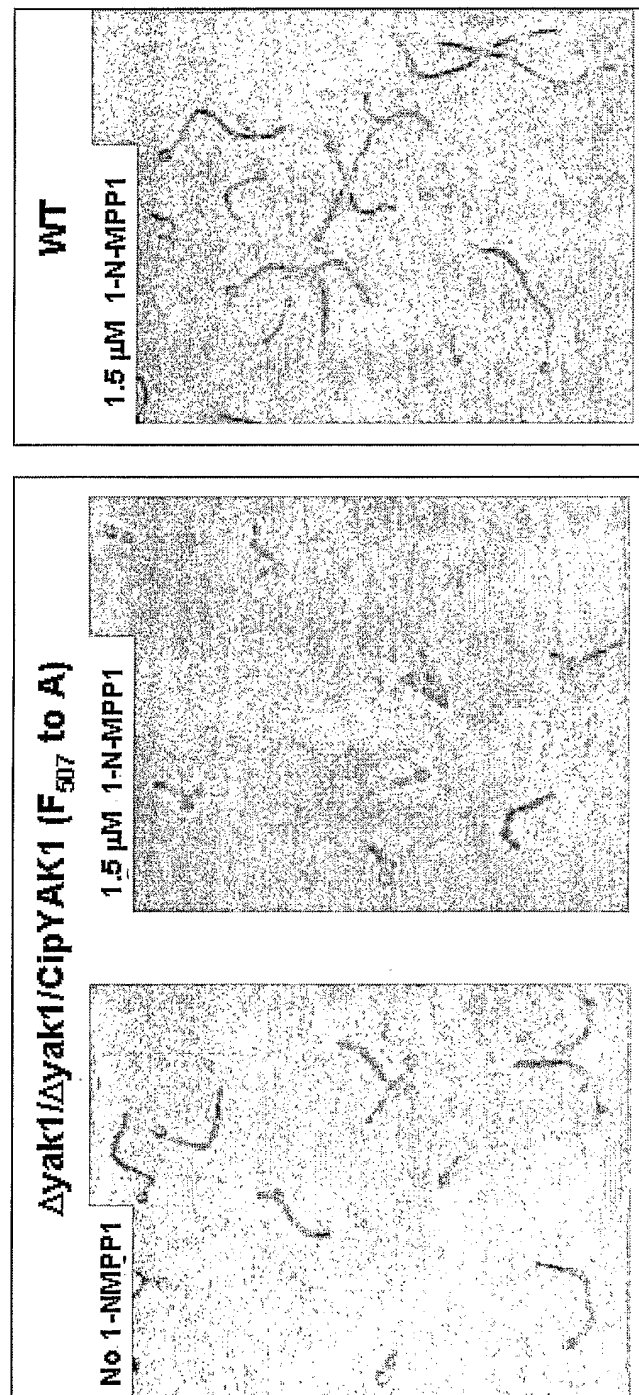


FIGURE 13

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MAYNNNNSSNYNYNFNRHNSIGGNWHLPPPPPPPTQLTSSGAYHHQQQQQHLOQHLSPNHP
NGQDILSSSDSQFLSQLKQNPQIPGFRNPWFSSQQQNSTPNMLSSSSSSASSSSLSQSPTKSHQIP
LLQQHQPPLLNKRLSFTNNLPITYEHSSSSSSNNSSSSNGNGNNNNNDNNTNVPPYIHP
TRADNPFNHYFATQEVTLGSNDRRMSAAVDGTHYNPYGFNQQLNAYPSGPAGIAGTQYLNNP
NLGPLVNaNRRSSVGVLPNYRQQQQQVQDQSTAAAAAAYYLPARLGRSTLIVQY
QQYQQALHNQRITKHKNPAPKARKIYNKLDLTPKFHQHPKYRRCINSIHSIPVNALSIYLT
ESYSLCQPRKFQYSKSTNPKRVLTKEPLEPKYNNGYDNEDSDYILYVNDVLGSEEGKKYMVLD
LLGSGTFGQVVKCQNLNNQTVCAVKVIKSKPAYMNQSLTEVRLLLEFLNANS DGKNFIRLLDT
FMHKEHLCLVFEILASNLIELIKQNFQGLNMKLVKLLTKQLLDSMAQLKNFQMIHCDLKE
NILLCQPDKNIKVIDFGSACFTRNTIYTIQSRFYRSPEVILGLPYTESIDMWSLGCIVGE
LFLGLPMFPGTSEYNQIFKIVDMLGPPPRHMIIEVGKNSFNFFKKKVNTTTTTINNNTTSE
TKPIYELKSFDEYCFLEYKRQKQEGATSTNNNTNSSSSSNHHNNNHKKKEQPNKNYFKHKL
LKDIIINYKLPSKKMTNSMIEKEYHDRLLLIDFLTQVLNLNPLERLTPQEALKHPFIIDVNT
TDL

FIGURE 14

MNLSSFTPSLRWLI IWTS LIPLLLAKDDYSSSLSNNNLGWTDPTFPLGCSPNVTTPKNGLS
MELYSYDYLKSGSNPCWDAAAYLDPNYPRTYGKSHRLLAKVENVAGNINFYYHAPMGCTSLFD
TLPQAYNYRTPLTMTNFTMLLYGYFKPKVTGYHTFTISADDLLFVNFGAGNAFDCKRESSA
DDFGNYQAYAVWGSQTAKDDLTVHLDAGLYPIRIFFNNRDNDGALSLLTKTESDSNPVIDF
SDYFYSFDDTKDGCPLVSYDTSCASVDSSTIIGVDYLTETPNKNLVPITRTIYHLGIPCTL
STTGKLCSTGFYDPLADSCVPTTKLSTLITSEPSSESSSETLYYSSSRSPSSSVSYSEHTSI
NSSSPISSITNSTPSLSSQYTSTKFSSITNSTKSPYSPSRSSSSSDISSETNSESSVYTLPS
SSKPITSKTYQKNSTGVMSTSTDVKSTTLCSSVPTSKFGSSRSLESSVIDSTSSFVNNTNHQ
SFTTESGTTSVNPSNLSTQISRMSSFSTFAKATTKISSSVQTSSHILQSPTTVSYMDHTRNR
TEASCSLDITNKLLFQQCAFNSVPEFHSKTQMSRSQIPFATYTTSSDKSVYYSSSGSASGIM
VSIESSIGFSISKTNSENTKNPHSIIILQIQSEEAFTNYATTTETSSKFKLADNNDSTNVGNI
QIETIIQGSGLVLTMSGATVLFVFFIVQLFGMF

FIGURE 15

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MNLSSTPSLRWLI IWTS LIPLLLAKDDYSSFLSNNNLGWTDPTFPLGCSPNVTTPKNGLS
MELYSYDLRKNGSYPCWDAAYLDPNYPRTGYKSHRLLAKVDGVTGNINFYYHATKGCTPELD
HLPASYNYPKPLTMTNFTMLLYGYFKPKVTGYHTFTISADDLLFVNFGAGNAFDCKRESSA
DDFGNYQAYAVWGSQTAKDDLT VHLDAGLYYPIRIFFNREYDAELSLTLKTESDSNPVIDF
SDYFYSFDDTKDGCPGLVSYDTSCASVDSSTIIGVDYLTETPNTNLVPITKTIYHLGIPCTL
STTGTL CSTGFYDPLADSCVPTTTVSTLITSEPSSSESSETLYYSSSRSPSSSVSYSEHTSI
NSSSPISSITNSTPSLSSQYTSTKFSITNSTKSSYSPSRSSSSSDISSETNSDSSVYTL PSS
SKPITSKTYQKNSTGVMSTSTDVKSTTLCSSVPTSKFGSSRSLESSVIDSTSSFVNNTNHQS
FTTESGTTSVNSSNLSTQISRMSSFSTFDKATTKISSSVQTSSHILQSPTTVSYMDHTRDRT
EASC SL DITNKLLFQQCAFNSVPEFQSKTQMSRSQIPFATYTTSSDKSVYYSRGSSSGIMV
SIESSIGFSISKTNSENTKNPQSIILQVQSEEAFTNYATTTETSSKSKLADNND SANVGNIQ
IETIIQGS GSDLVTMTGATV LFFFIVQLFGMF

FIGURE 16

LHLTPKFHQHPKYRRC SINSIHISPVNALSIYLTESYSLCQPRKFQYSKSTNPKRVLT KP
SEPKYNNGYDNEDSDYILYVNDVLGSEEGKKYMVLDLLGSGTFGQVVKCQNLNNQTVCAV
KVIKSKPAYMNQSLTEVRLLEFLNANS DGKNFIRLLDTFMHKEHLCLVFEILASNLYELI
KQNQFQGLNMKLVKLLTKQLLDSMAQLKNFQMIHCDLKPENILLCQPDKNIKVIDFGSA
CFTRNTIYTYIQSRFYRSPEVILGLPYTESIDMWSLGCIVGELFLGLPMFPGTSEYNQIF
KIVDMLGPPPRHMI EVGKNSFNFFKKKVNTTTTTINNNNNNTSETKPIYELKSFDEYCQF
LEYKRQKQEGATSTTNNTN SSSSNHHNNNHKKEQPNKNYFKHKLLKDIIINYKLPSKK
MTNSMIEKEYHDRLLLIDFLTKVLN LNPLERLTPQEAL KHPFIIDVNTTDL

FIGURE 17

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ATGGCATATAATAACAATAACAACAGTTCTAATTATAATTATAATTTTAATCGACACAATTC
TATAGGAGGAAATTGGCATTACCTCCACCACCACCTCCACCAACTCAACTTACTTCTAGTG
GTGCATACCACCACCAACAACAACAACAGCACCTGCAGCAACATTTATCACCTAATCATCCT
AATGGCCAAGATATTTTATCATCATCAGATTCTCAATTCCTGTCACAATTAAACAAAATCA
AATTCCTGGATTCCGTAACCCTTGGTTTTCTCAACAACAATAATTCTACCCCTAATATGTTAT
CATCTTCATCATCTGCATCATCTTCATTATCTCAATCACCGACTAAATCACATCAAATACCA
TTATTACAACAACATCAACCTCCATTACTAAATAAACGATTATCATTTACAAATAATTTACC
AATAACTTATGAACATGAAAGTAGTAGTAATAGTAGTAGTAACAACAACAGCAGCAGCAATG
GCAATGGCAACAATAATAACAATGATAATAATACTAATGTACCACCATATATACATCCACCA
ACTCGTGCTGATAATCCATTTAATCATTATTTTGCCACTCAAGAAGTTACTTTGGGTAGTAA
TGACCGAAGAATGTCGGCTGCTGTAGATGGAACCTCATTATAATCCATATGGATTCAATCAAC
AACTTAATGCTTATCCTTCTGGTCCTGCTGGAATTGCTGGTACTCAATATCTTAATAATCCT
AATTTGGGTCCACTGGTAAATGCCAATAGAAGATCATCTGTAGGGGTATTACCCAATTATTA
TCGTCAACAACAACAACAAGTTCAAGATCAGTCTACTGCTGCTGCTGCTGCTGCTGCTGCTG
CGGCAGCGGCATATTATCTTCCACCAGCTAGATTGGGAAGATCAACTCTGATTGTTCAATAT
CAACAATATCAACAAGCATTACACAATCAAAGAATAACTAAACATAAGAACCCTGCACCCAA
GGCAAGGAAAATATATAATAAATTGGATTTAACGCCTAAATTCATCAACACCCTAAATATA
GACGATGTTCTATAAATTCATATATCACCAGTCAATGCCTTATCGATATATTTAACA
GAATCATATAGTTTTATGTCAACCAAGAAAATTCCAATATTCGAAATCAACTAATCCTAAACG
AGTATTAATAAACCCTGGAACCTAAATATAATAATGGGTATGATAATGAAGATAGTGATT
ATATTTTATATGTTAATGATGTATTAGGAAGTGAAGAAGGGAAAAAATATATGGTATTAGAT
TTATTAGGTTTACGGTACATTTGGACAAGTAGTTAAATGTCAAATCTTAATAATCAAATGT
TTGTGCTGTTAAAGTGATCAAATCGAAACCAGCATATATGAATCAATCATTAACTGAAGTTC
GATTATTAGAAATTTTAAATGCTAATAGTGATGGGAAAAATTTTCATTCGATTATTGGATACA
TTTATGCATAAAGAACATCTTTGTTTAGTATTTGAAATCTTGGCATCGAATTTATATGAATT
AATTAAACAAAATCAATTTCAAGGTCTTAATATGAAATTAGTTAAATTTATTAATAAACAAT
TGTTAGATTCAATGGCTCAATTGAAAAATTTCCAATGATTCATTGTGATTTAAAACCAGAA
AATATCTTATTATGTCAACCCGATAAACCCAATATAAAAGTCATTGATTTTGGTAGTGCTTG
TTTCACAAGAAATACTATATATACTTATATTCAATCAAGATTTTATCGATCACCAGAAGTAA
TATTAGGTTTACCTTATACTGAATCAATTGATATGTGGTCATTAGGTTGTATCGTGGGGGAA
TTATTTTTAGGTTTACCAATGTTTCCTGGAACCTCAGAATATAATCAAATTTTTTAAATTTGT
TGATATGTTGGGTCTCCACCAAGACATATGATTGAAGTTGGGAAAAATTCATTCAATTTCT
TTAAAAAGAAAGTCAACACCACCACCACCACCATCAACAACAATAATAATAACTTCAGAA
ACAAAACCAATTTATGAACTTAAATCATTGATGAATATTGTCAATTTTGAATATAAACG
ACAAAAACAAGAAGGTGCTACATCTACAACCAACAACAATACTAATAGTAGTAGTAGTAGTA
ATCATCATAATAATAATCATAAAAAGGAACAACCAATAAAAAATTATTTTAAACATAAATTA
TTAAAAGATATTATTATTAATTATAAATTACCTTCAAAAAAATGACAAATTCATGATTGA
AAAAGAATATCATGATCGATTATTATTAATTGATTTTTTAACTAAAGTACTCAATTTAAATC
CATTAGAAAGATTAACACCTCAAGAAGCTTTAAACATCCATTTATTATTGATGTTAATACC
ACTGATTTATAA

FIGURE 18

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ATGAATTTATCATCTTTTACACCATCTCTAAGATGGTTGATTATCTGGACTTCTTTGATACC
TTTACTATTGGCAAAGGATGACTATTCTTCCTCCTTGTCCAATAATAATTTAGGATGGACGG
ACCCTACTGAATTTCTCTTGGATGTTACCAAATGTTACAACACCAAAAAATGGATTGTCTG
ATGGAACTTTATTCTTATGATTATTTGAAATCAGGATCGAATCCATGTTGGGATGCAGCATA
TCTAGATCCGAATTATCCTCGAACAGGCTATAAATCACACAGACTTCTTGCAAAAGTTGAAA
ATGTTGCTGGAAATATAAATTTTTATTATCATGCCCAATGGGGTGTACTTCGCTGTTTGAT
ACATTACCACAAGCTTATAACTATCGTACACCATTGACAATGACAAATTTTACTATGTTACT
CTATGGCTACTTTAAACCAAAAGTAACCGGTTATCATACGTTTACAATTTTCGGCCGACGATT
TACTGTTTGTGAATTTTGGGGCTGGTAACGCGTTTGATTGTTGTAAAAGGGAATCCAGCGCT
GATGACTTTGGTAATTATCAGGCTTATGCCGTATGGGGTCTCAAACAGCTAAGGATGATTT
AACAGTTCATTTAGATGCAGGTCTATACTATCCAATTAGAATTTTCTTTAATAATAGAGACA
ATGATGGTGCATTAAAGTCTAACATTAAAACTGAGTCAGATTTCGAACCCTGTAATAGACTTC
TCTGACTATTTCTATTCATTTGATGATACAAAAGATGGTTGTCCAGGCTTAGTTAGTTATGA
TACTTCGTGTGCCTCAGTTGACTCATCTACAATCATCGGTGTTGACTACCTAACAGAAACAC
CAAACAAAAATCTAGTTCCAATCACTAGAACAATTTACCATCTAGGAATTCCTTGTACTCTA
TCCACAACAGGTAAATTATGCAGCACCGGGTTTATGACCCGTTAGCAGATAGTTGTGTGCC
AACAAACAAAACCTCTCGACTCTGATAACATCAGAACCAAGTTCAGAGTCTTCTTCTGAAACTT
TATACTACTCTTCGAGTAGAAGCCCTTCTTCAAGTGTTTCATATTCGGAACATACTAGTATC
AACAGCAGCAGTCCTATCTCATCGATCACCAATAGTACTCCTTCATTATCTTCTCAATATAC
TTCTACCAAGTTTTCTTCAATAACCAACTCTACGAAGTCACCATATTCACCAAGTCGTTCTA
GTTTCATCATCTGATATATCTTCTGAAACAACTCTGAAAGTTCAGTCTATACACTACCAAGT
AGTAGCAAACCGATAACATCTAAAACCTACCAGAAGAATAGTACTGGGGTTATGTCTACGAG
CACAGATGTGAAATCAACAACCTTTATGCAGTTCGGTTCCTACAAGTAAATTTGGTTCAAGTA
GGTCATTAGAATCGTCAGTAATTGATTCAACATCATCCTTTGTCAATAATACAAACCATCAA
TCATTTACTACAGAATCAGGTACCACTTCAGTTAACCCAAGCAATCTATCCACACAGATTTT
AAGGATGTCAAGTTTTTCTACATTTGCTAAGGCCACTACAAAAATAAGTTCATCTGTACAAA
CCTCCAGTCATATTCTACAAAGCCCAACAACAGTTTCATATATGGATCACACACGTAACAGA
ACAGAGGCTAGCTGTTCTTTGGACATTACGAATAAGCTTCTGTTCCAACAGTGTGCTTTTAA
TTCAGTTCCAGAGTTTCATAGCAAAACGCAGATGTCTAGATCTCAAATTCCTTCGCAACTT
ACACAACCTTCCTCAGACAAATCTGTTTATTACTCTAGCAGTGGGAGTGCTTCAGGTATAATG
GTTTCTATAGAGAGTTCATTGGTTTTTCCATTTCAAAAATAATTCTGAGAATACCAAGAA
CCCTCACTCTATTATTTTGCAAATACAATCTGAAGAAGCATTCACTAATTATGCTACTACAA
CAGAGACATCTTCAAAATTTAACTGGCAGACAATAATGATTCTACTAATGTTGGAAACATA
CAAATTGAAACTATTATCCAAGGTTCTGGTTCTGATCTTGTGACGATGTCTGGGGCAACAGT
ATTGTTCTTTTTTCATCGTGCAGCTATTTGGCATGTTCTAG

FIGURE 19

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ATGAATTTATCATCTTTTACACCATCTCTAAGATGGTTGATTATCTGGACTTCTTTGATACC
TTTACTATTGGCAAAGGATGACTATTCTTCCTTCTTGTCCAATAATAATTTAGGATGGACGG
ACCCTACTGAATTTCTTCTTGGATGTTACCAAATGTTACAACACCAAAAAATGGATTGTCG
ATGGAACCTTTATTCCCTATGATTTACGGAAGAATGGTTCGTACCCATGTTGGGATGCCGCATA
TTTAGATCCCAATTACCCTAGAAGTGGCTATAAAAGCCACAGACTTCTAGCAAAAGTTGATG
GTGTTACCGGTAATATAAATTTCTATTATCATGCAACCAAAGGATGCACACCCGAATTAGAT
CATTTACCGGCAAGCTATAATTACCCTAAGCCATTAACAATGACAAATTTTACTATGTTACT
CTATGGCTACTTTAAACCAAAAAGTAACCGGTTATCATACGTTTACAATTTTCGGCCGACGATT
TACTGTTTGTGAATTTTGGGGCTGGTAACGCGTTTGATTGTTGTAAAAGGGAATCCAGCGCT
GATGACTTTGGTAATTATCAGGCTTATGCCGTATGGGGTCTCAAACAGCTAAGGATGATTT
AACAGTTCATTTAGATGCAGGTCTATACTATCCAATTAGAATTTTCTTTAATAATAGAGAAT
ATGATGCTGAATTAAGTCTAACATTA AAAACTGAGTCAGATTCTGAACCTGTAATAGACTTC
TCTGACTATTTCTATTCTATTTGATGATACAAAAGATGGTTGTCCAGGCTTAGTTAGTTATGA
TACTTCGTGTGCCTCAGTTGACTCATCTACAATCATCGGTGTTGACTACCTAACAGAAACAC
CAAACACAAATCTAGTTCCAATCACTAAAACAATTTACCATCTAGGAATTCCTTGTACTCTA
TCCACAACAGGTACATTATGCAGCACCGGGTTTTATGACCCGTTAGCAGATAGTTGTGTGCC
AACAACAACAGTCTCGACTCTGATAACATCAGAACCAAGTTCAGAGTCTTCTTCTGAAACTT
TATACTACTCTTCGAGTAGAAGCCCTTCTTCAAGTGTTCATATTCGGAACATACTAGTATC
AACAGCAGCAGTCCTATCTCATCGATCACCAATAGTACTCCTTCATTATCTTCTCAATATAC
TTCTACCAAGTTTTCAATAACCAACTCTACGAAGTCATCATATTCACCAAGTCGTTCTAGTT
CATCATCTGATATATCTTCTGAAACAAACTCTGATAGTTCAGTCTATACACTACCAAGTAGT
AGCAAACCGATAACATCTAAACCTACCAGAAGAATAGTACTGGGGTTATGTCTACGAGCAC
AGATGTGAAATCAACAACCTTTATGCAGTTCGGTTCCTACAAGTAAATTTGGTTCAAGTAGGT
CATTAGAATCGTCAGTAATTGATTCAACATCATCTTTTGTCAATAATACAAACCATCAATCA
TTTACTACAGAATCAGGTACCACTTCAGTTAACTCAAGCAATCTATCCACACAGATTTCAAG
GATGTCAAGTTTTTCTACATTTGATAAGGCCACTACAAAAATAAGTTCGTCTGTACAACTT
CCAGTCATATTCTACAAAGCCCAACAACAGTTTCATATATGGATCACACACGTGACAGAACA
GAGGCTAGCTGTTCTTTGGACATTACGAATAAGCTTCTGTTCCAACAGTGTGCTTTTAATTC
AGTTCCAGAGTTTCAAAGCAAAACGCAGATGTCTAGATCTCAAATTCCTTCGCAACTTACA
CAACTTCCTCAGACAAATCTGTTTATTACTCTAGAAGTGGGAGTTCTTCAGGTATAATGGTT
TCTATAGAGAGTTCCATTGGTTTTTCCATTTCAAAAATAATTCTGAGAATACCAAGAACCC
TCAATCTATTATTTTGAAGTTCAATCTGAAGAAGCATTCCTAATTATGCTACTACAACAG
AGACATCTTCAAATCTAAACTGGCAGACAATAATGATTCTGCTAATGTTGGAAACATACAA
ATTGAAACTATTATCCAAGGTTCTGGTTCTGATCTTGTGACGATGACTGGGGCAACAGTATT
GTTCTTTTTCATCGTGCAGCTATTTGGTATGTTCTAG

FIGURE 20

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CTGCATTTAACGCCTAAATTCCATCAACACCCTAAATATAGACGATGTTCTATAAAATTCAAT
TCATATATCACCAGTCAATGCCTTATCGATATATTTAACAGAATCATATAGTTTATGTCAAC
CAAGAAAATTCCAATATTCGAAATCAACTAATCCTAAACGAGTATTAATAAACCCTGGAA
CCTAAATATAATAATGGGTATGATAATGAAGATAGTGATTATATTTTATATGTTAATGATGT
ATTAGGAAGTGAAGAAGGGGAAAAAATATATGGTATTAGATTTATTAGGTTTCAGGTACATTTG
GACAAGTAGTTAAATGTCAAAATCTTAATAATCAAACCTGTTTGTGCTGTTAAAGTGATCAAA
TCGAAACCAGCATATATGAATCAATCAATTAACCTGAAGTTGATTATTAGAATTTTTTAAATGC
TAATAGTGATGGGAAAAATTTTCATTGATTATTGGATACATTTATGCATAAAGAACATCTTT
GTTTAGTATTTGAAATCTTGGCATCGAATTTATATGAATTAATTAACAAAATCAATTTCAA
GGTCTTAATATGAAATTAGTTAAATTATTAACATAAACAATTGTTAGATTCAATGGCTCAATT
GAAAAATTTCCAAATGATTCAATTGTGATTTAAACCAGAAAATATCTTATTATGTCAACCCG
ATAAACCCAATATAAAAGTCATTGATTTTGGTAGTGCTTGTTTCACAAGAAATACTATATAT
ACTTATATTCAATCAAGATTTTATCGATCACCAGAAGTAATATTAGGTTTACCTTATACTGA
ATCAATTGATATGTGGTCATTAGGTTGTATCGTGGGGGAATTATTTTTAGGTTTACCAATGT
TTCCTGGAACCTCAGAATATAATCAAAATTTTAAATTTGTTGATATGTTGGGTCCTCCACCA
AGACATATGATTGAAGTTGGGAAAAATTCATTCAATTTCTTTAAAAAGAAAGTCAACACCAC
CACCACCACCATCAACAACAATAATAATAACTTCAGAAACAAAACCAATTTATGAACCTTA
AATCATTTTGATGAATATTGTCAATTTTTAGAATATAAACGACAAAAACAAGAAGGTGCTACA
TCTACAACCAACAACAATACTAATAGTAGTAGTAGTAATCATCATAATAATAATCATAA
AAAGGAACAACCAATAAAAAATTATTTTAAACATAAATTATTTAAAGATATTATTATTAATT
ATAAATTACCTTCAAAAAAATGACAAATTCATGATTGAAAAAGAATATCATGATCGATTA
TTATTAATTGATTTTAACTAAAGTACTCAATTTAAATCCATTAGAAAGATTAAACACCTCA
AGAAGCTTTAAACATCCATTTATTATTGATGTTAATACCACTGATTTATAAgaataagaat
aagaataacaatgtggagggagggggaggttgaccagtattaagttttatactccttaatga
tacttttttaattaattttgtttatgtaacaatccttggtttttgttaagaactgaatatgggtt
tatatagcatcatataaattttccttaataggaataggaataagaataggaatatgaataatag
gaataggaatagtcataataataatgaattgaattgggtatatcaacttgacaattaaaagaa
tggtcgtgtagaattgtaaattttcccaaaaaaaaaaagaaaaaaaatccttcaactctttca
gaagaagaacactggaaatcaacaaagtgtcaaccattcaaccatttaaccatttaactaac
cctcaataatatctacatccttgctattgaattggaatagattatcactacattgttttataa
tagagaataatcattatcattatcattatcattatcatttagttttcagaaaagactacacct
tcaattaaatctgtaaatataatcaacaacattatttaaaaaaaataaattattgatcac
aataaaaacccaataattatcaatcctaattacaatctacattttggacgaaaacaataaatca
tttattaatactatcatttttttaaaaccattatagaacatatatatatatatatatcac
acacacattatcatacttactagcaacctatcattatgaatgtatcaactagtgccttagga
gtaagaagaagaaaagtaacaacacaaaacattgatgatgaagaaaattctgcggtattaaa
attaggaccagaattccaactaaatcaaatcacaatgatggagaagaacaacaattaatg
cattaaatttatccgaagcaagattattaattcgagcagcattaaaagaagaaaacataat
aaaaataaaaaataaaaaccagaaaaaagggaggttaataataataatagtagtagtatt

FIGURE 21

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MAYNNNNNSSNYNYNENRHN SIGGNWHLPPPPPPPTQLTSSGAYHHQQQQQHLLQOHLSPNHP
NGQDILSSSDSQFLSQLKQIQIPGFRNPWFSSQQQNSTPNMLSSSSSSASSSLSQSPTKSHQIP
LLQQHQPPLLNKRLSFTNNLPITYEHSSSNSSSNSSNGNGNNNNNDNNTNVPPYIHP
TRADNPFNFHYFATQEVTLGSNDRMSAAVDGTHYNPYGFNQQLNAYPSGPAGIAGTQYLNNP
NLGPLVNAVNRSSVGVLPNYYRQQQQQQVQDQSTAAAAAAYYLPPARLGRSTLIVQY
QQYQQALHNQRITKHKNPAPKARKIYNKLDLTPKFHQHPKYRRCINSIHISPVNALSIIYLT
ESYSLCQPRKFQYSKSTNPKRVLTKEPKYNNGYDNEDSDYILYVNDVLGSEEGKKYMLVD
LLGSGTFGQVVKCQNLNNQTVCAVKVIKSKPAYMNQSLTEVRLLLEFLNANS DGKNFIRLLDT
FMHKEHLCLVAEILASNLIELIKQNFQGLNMKLVKLLTKQLLDSMAQLKNFQMIHCDLKPE
NILLCQPDKPNIKVIDFGSACFTRNTIYTYIQSRFYRSPEVILGLPYTESIDMWSLGCIVGE
LFLGLPMFPGTSEYNQIFKIVDMLGPPPRHMEVKGNSFNFFKKKVNTTTTTINNNNNNTSE
TKPIYELKSFDEYCFLEYKRQKQEGATSTNNNTNSSSSSNHHNNNHKKEQPNKNYFKHKL
LKDIIINYKLPSKMTNSMIEKEYHDRLLLIDFLTKVLNPLERLTPQEALKHPFIIDVNT
TDL

FIGURE 22

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MVQIYYNENQALEPMQHNQANGIWKASYMDEANADSKRFAFTNPWGNEKIDEDDEAMSYNYSS
ANSTADVDMNSDMYKRRKSSLVPPSRAAAPNPFQYETMQNSTGTAQRAMYPESSAYQQQRQ
IMVPHQQQGQLASRFNSRFVPSLYNPQEFQRRQSLATAQFSTPSSLHGSASNQAFNTGSGNN
GVNLNYASANVTNNSSSTYLSAMTPNVPMEGNIPLASPHRRLSAFPTSGTTNSSLQPPFKQ
LRRDEGSPRQIVIPQMHRCHSKNDLQPCINPNPKFRRASLHSNTISPLIGLTKSLLTTYALC
SPDFTYQTSKNPKRVLTKEPSEGKYNNGYDNVNSDYILYVNDVLGTEQNRKYLVLDDILGQGT
GQVVKCQNLQTKELIAVKVVKSRSEYLNQSISEAKILELLNEKIDPNNKHHFLRMHDSFVHK
NHLCLVFELLSNNLYELLKQNKFHGLSIQLIRTFITQILDLCVLKDSKLIHCDLKPENILL
CSPDKPDLKVIDFGSSCEETRTVYTYIQSRFYRAPEIILGIPYSTSIDMWSLGCIVAEFLG
IPIFPGSSEFNQLTRIITTLGYPPTWMIDMGKSSHKFLKKLNTLNLDMTNINDVEAQNNNAN
YTTANRMQKNQPTAKYRLKTIEEFNRDNNASEKVGKQYFKWALLEDIIRNYRIHKNIQKSPE
LIEQEMKNRECLIHFLKGILNPLERWTPQQAAMHPFITQQPFTGKWFPPGSFNTMNTSNF
GSQPRQEGNNSNMKPNSQEGPTKEFTNLKIIED

FIGURE 23

23/23

atggtacagatatataatgagaaccaagcggttagaaccgatgcaacacaaccaagctaa
cggcatatggaaggcctcctacatggacgaggccaatgcagactctaagaggttcgcattca
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FIGURE 24

SEQUENCE LISTING

<110> INSTITUT PASTEUR

<120> POLYPEPTIDES INVOLVED IN CANDIDA BIOFILM FORMATION
AND USES THEREOF

<130> D23940-348752

<160> 54

<170> PatentIn version 3.3

<210> 1

<211> 809

<212> PRT

<213> Candida albicans

<220>

<223> Yak1 amino acid sequence

<400> 1

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Asn Arg His Asn Ser Ile Gly Gly Asn Trp His Leu Pro Pro Pro Pro
          20          25          30
Pro Pro Pro Thr Gln Leu Thr Ser Ser Gly Ala Tyr His His Gln Gln
          35          40          45
Gln Gln Gln His Leu Gln Gln His Leu Ser Pro Asn His Pro Asn Gly
          50          55          60
Gln Asp Ile Leu Ser Ser Ser Asp Ser Gln Phe Leu Ser Gln Leu Lys
65          70          75          80
Gln Asn Gln Ile Pro Gly Phe Arg Asn Pro Trp Phe Ser Gln Gln Gln
          85          90          95
Asn Ser Thr Pro Asn Met Leu Ser Ser Ser Ser Ser Ala Ser Ser Ser
          100          105          110
Leu Ser Gln Ser Pro Thr Lys Ser His Gln Ile Pro Leu Leu Gln Gln
          115          120          125
His Gln Pro Pro Leu Leu Asn Lys Arg Leu Ser Phe Thr Asn Asn Leu
          130          135          140
Pro Ile Thr Tyr Glu His Glu Ser Ser Ser Asn Ser Ser Ser Asn Asn
145          150          155          160
Asn Ser Ser Ser Asn Gly Asn Gly Asn Asn Asn Asn Asp Asn Asn
          165          170          175
Thr Asn Val Pro Pro Tyr Ile His Pro Pro Thr Arg Ala Asp Asn Pro
          180          185          190
Phe Asn His Tyr Phe Ala Thr Gln Glu Val Thr Leu Gly Ser Asn Asp
          195          200          205
Arg Arg Met Ser Ala Ala Val Asp Gly Thr His Tyr Asn Pro Tyr Gly
          210          215          220
Phe Asn Gln Gln Leu Asn Ala Tyr Pro Ser Gly Pro Ala Gly Ile Ala
225          230          235          240
Gly Thr Gln Tyr Leu Asn Asn Pro Asn Leu Gly Pro Leu Val Asn Ala
          245          250          255
Asn Arg Arg Ser Ser Val Gly Val Leu Pro Asn Tyr Tyr Arg Gln Gln
          260          265          270
Gln Gln Gln Gln Val Gln Asp Gln Ser Thr Ala Ala Ala Ala Ala Ala
          275          280          285
Ala Ala Ala Ala Ala Tyr Tyr Leu Pro Pro Ala Arg Leu Gly Arg Ser

```

290	295	300
Thr Leu Ile Val Gln	Tyr Gln Gln Tyr Gln	Gln Ala Leu His Asn Gln
305	310	315
Arg Ile Thr Lys His	Lys Asn Pro Ala Pro	Lys Ala Arg Lys Ile Tyr
325	330	335
Asn Lys Leu Asp Leu	Thr Pro Lys Phe His	Gln His Pro Lys Tyr Arg
340	345	350
Arg Cys Ser Ile Asn	Ser Ile His Ile Ser	Pro Val Asn Ala Leu Ser
355	360	365
Ile Tyr Leu Thr Glu	Ser Tyr Ser Leu Cys	Gln Pro Arg Lys Phe Gln
370	375	380
Tyr Ser Lys Ser Thr	Asn Pro Lys Arg Val	Leu Thr Lys Pro Leu Glu
385	390	395
Pro Lys Tyr Asn Asn	Gly Tyr Asp Asn Glu	Asp Ser Asp Tyr Ile Leu
405	410	415
Tyr Val Asn Asp Val	Leu Gly Ser Glu Glu	Gly Lys Lys Tyr Met Val
420	425	430
Leu Asp Leu Leu Gly	Ser Gly Thr Phe Gly	Gln Val Val Lys Cys Gln
435	440	445
Asn Leu Asn Asn Gln	Thr Val Cys Ala Val	Lys Val Ile Lys Ser Lys
450	455	460
Pro Ala Tyr Met Asn	Gln Ser Leu Thr Glu	Val Arg Leu Leu Glu Phe
465	470	475
Leu Asn Ala Asn Ser	Asp Gly Lys Asn Phe	Ile Arg Leu Leu Asp Thr
485	490	495
Phe Met His Lys Glu	His Leu Cys Leu Val	Phe Glu Ile Leu Ala Ser
500	505	510
Asn Leu Tyr Glu Leu	Ile Lys Gln Asn Gln	Phe Gln Gly Leu Asn Met
515	520	525
Lys Leu Val Lys Leu	Leu Thr Lys Gln Leu	Leu Asp Ser Met Ala Gln
530	535	540
Leu Lys Asn Phe Gln	Met Ile His Cys Asp	Leu Lys Pro Glu Asn Ile
545	550	555
Leu Leu Cys Gln Pro	Asp Lys Pro Asn Ile	Lys Val Ile Asp Phe Gly
565	570	575
Ser Ala Cys Phe Thr	Arg Asn Thr Ile Tyr	Thr Tyr Ile Gln Ser Arg
580	585	590
Phe Tyr Arg Ser Pro	Glu Val Ile Leu Gly	Leu Pro Tyr Thr Glu Ser
595	600	605
Ile Asp Met Trp Ser	Leu Gly Cys Ile Val	Gly Glu Leu Phe Leu Gly
610	615	620
Leu Pro Met Phe Pro	Gly Thr Ser Glu Tyr	Asn Gln Ile Phe Lys Ile
625	630	635
Val Asp Met Leu Gly	Pro Pro Pro Arg His	Met Ile Glu Val Gly Lys
645	650	655
Asn Ser Phe Asn Phe	Phe Lys Lys Lys Val	Asn Thr Thr Thr Thr
660	665	670
Ile Asn Asn Asn Asn	Asn Asn Thr Ser Glu	Thr Lys Pro Ile Tyr Glu
675	680	685
Leu Lys Ser Phe Asp	Glu Tyr Cys Gln Phe	Leu Glu Tyr Lys Arg Gln
690	695	700
Lys Gln Glu Gly Ala	Thr Ser Thr Thr Asn	Asn Asn Thr Asn Ser Ser
705	710	715
Ser Ser Ser Asn His	His Asn Asn Asn His	Lys Lys Glu Gln Pro Asn
725	730	735
Lys Asn Tyr Phe Lys	His Lys Leu Leu Lys	Asp Ile Ile Ile Asn Tyr
740	745	750
Lys Leu Pro Ser Lys	Lys Met Thr Asn Ser	Met Ile Glu Lys Glu Tyr
755	760	765
His Asp Arg Leu Leu	Leu Ile Asp Phe Leu	Thr Lys Val Leu Asn Leu
770	775	780

Asn Pro Leu Glu Arg Leu Thr Pro Gln Glu Ala Leu Lys His Pro Phe
 785 790 795 800
 Ile Ile Asp Val Asn Thr Thr Asp Leu
 805

<210> 2

<211> 715

<212> PRT

<213> Candida glabrata

<220>

<223> Epa6 amino acid sequence

<400> 2

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 Thr Ser Leu Ile Pro Leu Leu Leu Ala Lys Asp Asp Tyr Ser Ser Ser
 20 25 30
 Leu Ser Asn Asn Asn Leu Gly Trp Thr Asp Pro Thr Glu Phe Pro Leu
 35 40 45
 Gly Cys Ser Pro Asn Val Thr Thr Pro Lys Asn Gly Leu Ser Met Glu
 50 55 60
 Leu Tyr Ser Tyr Asp Tyr Leu Lys Ser Gly Ser Asn Pro Cys Trp Asp
 65 70 75 80
 Ala Ala Tyr Leu Asp Pro Asn Tyr Pro Arg Thr Gly Tyr Lys Ser His
 85 90 95
 Arg Leu Leu Ala Lys Val Glu Asn Val Ala Gly Asn Ile Asn Phe Tyr
 100 105 110
 Tyr His Ala Pro Met Gly Cys Thr Ser Leu Phe Asp Thr Leu Pro Gln
 115 120 125
 Ala Tyr Asn Tyr Arg Thr Pro Leu Thr Met Thr Asn Phe Thr Met Leu
 130 135 140
 Leu Tyr Gly Tyr Phe Lys Pro Lys Val Thr Gly Tyr His Thr Phe Thr
 145 150 155 160
 Ile Ser Ala Asp Asp Leu Leu Phe Val Asn Phe Gly Ala Gly Asn Ala
 165 170 175
 Phe Asp Cys Cys Lys Arg Glu Ser Ser Ala Asp Asp Phe Gly Asn Tyr
 180 185 190
 Gln Ala Tyr Ala Val Trp Gly Ser Gln Thr Ala Lys Asp Asp Leu Thr
 195 200 205
 Val His Leu Asp Ala Gly Leu Tyr Tyr Pro Ile Arg Ile Phe Phe Asn
 210 215 220
 Asn Arg Asp Asn Asp Gly Ala Leu Ser Leu Thr Leu Lys Thr Glu Ser
 225 230 235 240
 Asp Ser Asn Pro Val Ile Asp Phe Ser Asp Tyr Phe Tyr Ser Phe Asp
 245 250 255
 Asp Thr Lys Asp Gly Cys Pro Gly Leu Val Ser Tyr Asp Thr Ser Cys
 260 265 270
 Ala Ser Val Asp Ser Ser Thr Ile Ile Gly Val Asp Tyr Leu Thr Glu
 275 280 285
 Thr Pro Asn Lys Asn Leu Val Pro Ile Thr Arg Thr Ile Tyr His Leu
 290 295 300
 Gly Ile Pro Cys Thr Leu Ser Thr Thr Gly Lys Leu Cys Ser Thr Gly
 305 310 315 320
 Phe Tyr Asp Pro Leu Ala Asp Ser Cys Val Pro Thr Thr Lys Leu Ser
 325 330 335
 Thr Leu Ile Thr Ser Glu Pro Ser Ser Glu Ser Ser Ser Glu Thr Leu
 340 345 350

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Tyr Tyr Ser Ser Ser Arg Ser Pro Ser Ser Ser Val Ser Tyr Ser Glu
    355                      360          365
His Thr Ser Ile Asn Ser Ser Ser Pro Ile Ser Ser Ile Thr Asn Ser
    370                      375          380
Thr Pro Ser Leu Ser Ser Gln Tyr Thr Ser Thr Lys Phe Ser Ser Ile
385                      390          395          400
Thr Asn Ser Thr Lys Ser Pro Tyr Ser Pro Ser Arg Ser Ser Ser Ser
    405                      410          415
Ser Asp Ile Ser Ser Glu Thr Asn Ser Glu Ser Ser Val Tyr Thr Leu
    420                      425          430
Pro Ser Ser Ser Lys Pro Ile Thr Ser Lys Thr Tyr Gln Lys Asn Ser
    435                      440          445
Thr Gly Val Met Ser Thr Ser Thr Asp Val Lys Ser Thr Thr Leu Cys
    450                      455          460
Ser Ser Val Pro Thr Ser Lys Phe Gly Ser Ser Arg Ser Leu Glu Ser
465                      470          475          480
Ser Val Ile Asp Ser Thr Ser Ser Phe Val Asn Asn Thr Asn His Gln
    485                      490          495
Ser Phe Thr Thr Glu Ser Gly Thr Thr Ser Val Asn Pro Ser Asn Leu
    500                      505          510
Ser Thr Gln Ile Ser Arg Met Ser Ser Phe Ser Thr Phe Ala Lys Ala
    515                      520          525
Thr Thr Lys Ile Ser Ser Ser Val Gln Thr Ser Ser His Ile Leu Gln
    530                      535          540
Ser Pro Thr Thr Val Ser Tyr Met Asp His Thr Arg Asn Arg Thr Glu
545                      550          555          560
Ala Ser Cys Ser Leu Asp Ile Thr Asn Lys Leu Leu Phe Gln Gln Cys
    565                      570          575
Ala Phe Asn Ser Val Pro Glu Phe His Ser Lys Thr Gln Met Ser Arg
    580                      585          590
Ser Gln Ile Pro Phe Ala Thr Tyr Thr Thr Ser Ser Asp Lys Ser Val
    595                      600          605
Tyr Tyr Ser Ser Ser Gly Ser Ala Ser Gly Ile Met Val Ser Ile Glu
    610                      615          620
Ser Ser Ile Gly Phe Ser Ile Ser Lys Thr Asn Ser Glu Asn Thr Lys
625                      630          635          640
Asn Pro His Ser Ile Ile Leu Gln Ile Gln Ser Glu Glu Ala Phe Thr
    645                      650          655
Asn Tyr Ala Thr Thr Thr Glu Thr Ser Ser Lys Phe Lys Leu Ala Asp
    660                      665          670
Asn Asn Asp Ser Thr Asn Val Gly Asn Ile Gln Ile Glu Thr Ile Ile
    675                      680          685
Gln Gly Ser Gly Ser Asp Leu Val Thr Met Ser Gly Ala Thr Val Leu
    690                      695          700
Phe Phe Phe Ile Val Gln Leu Phe Gly Met Phe
705                      710          715

```

<210> 3

<211> 714

<212> PRT

<213> Candida glabrata

<220>

<223> Epa7 amino acid sequence

<400> 3

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Met Asn Leu Ser Ser Phe Thr Pro Ser Leu Arg Trp Leu Ile Ile Trp
1          5          10          15

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 20 25 30
 Leu Ser Asn Asn Asn Leu Gly Trp Thr Asp Pro Thr Glu Phe Pro Leu
 35 40 45
 Gly Cys Ser Pro Asn Val Thr Thr Pro Lys Asn Gly Leu Ser Met Glu
 50 55 60
 Leu Tyr Ser Tyr Asp Leu Arg Lys Asn Gly Ser Tyr Pro Cys Trp Asp
 65 70 75 80
 Ala Ala Tyr Leu Asp Pro Asn Tyr Pro Arg Thr Gly Tyr Lys Ser His
 85 90 95
 Arg Leu Leu Ala Lys Val Asp Gly Val Thr Gly Asn Ile Asn Phe Tyr
 100 105 110
 Tyr His Ala Thr Lys Gly Cys Thr Pro Glu Leu Asp His Leu Pro Ala
 115 120 125
 Ser Tyr Asn Tyr Pro Lys Pro Leu Thr Met Thr Asn Phe Thr Met Leu
 130 135 140
 Leu Tyr Gly Tyr Phe Lys Pro Lys Val Thr Gly Tyr His Thr Phe Thr
 145 150 155 160
 Ile Ser Ala Asp Asp Leu Leu Phe Val Asn Phe Gly Ala Gly Asn Ala
 165 170 175
 Phe Asp Cys Cys Lys Arg Glu Ser Ser Ala Asp Asp Phe Gly Asn Tyr
 180 185 190
 Gln Ala Tyr Ala Val Trp Gly Ser Gln Thr Ala Lys Asp Asp Leu Thr
 195 200 205
 Val His Leu Asp Ala Gly Leu Tyr Tyr Pro Ile Arg Ile Phe Phe Asn
 210 215 220
 Asn Arg Glu Tyr Asp Ala Glu Leu Ser Leu Thr Leu Lys Thr Glu Ser
 225 230 235 240
 Asp Ser Asn Pro Val Ile Asp Phe Ser Asp Tyr Phe Tyr Ser Phe Asp
 245 250 255
 Asp Thr Lys Asp Gly Cys Pro Gly Leu Val Ser Tyr Asp Thr Ser Cys
 260 265 270
 Ala Ser Val Asp Ser Ser Thr Ile Ile Gly Val Asp Tyr Leu Thr Glu
 275 280 285
 Thr Pro Asn Thr Asn Leu Val Pro Ile Thr Lys Thr Ile Tyr His Leu
 290 295 300
 Gly Ile Pro Cys Thr Leu Ser Thr Thr Gly Thr Leu Cys Ser Thr Gly
 305 310 315 320
 Phe Tyr Asp Pro Leu Ala Asp Ser Cys Val Pro Thr Thr Thr Val Ser
 325 330 335
 Thr Leu Ile Thr Ser Glu Pro Ser Ser Glu Ser Ser Ser Glu Thr Leu
 340 345 350
 Tyr Tyr Ser Ser Ser Arg Ser Pro Ser Ser Ser Val Ser Tyr Ser Glu
 355 360 365
 His Thr Ser Ile Asn Ser Ser Ser Pro Ile Ser Ser Ile Thr Asn Ser
 370 375 380
 Thr Pro Ser Leu Ser Ser Gln Tyr Thr Ser Thr Lys Phe Ser Ile Thr
 385 390 395 400
 Asn Ser Thr Lys Ser Ser Tyr Ser Pro Ser Arg Ser Ser Ser Ser Ser
 405 410 415
 Asp Ile Ser Ser Glu Thr Asn Ser Asp Ser Ser Val Tyr Thr Leu Pro
 420 425 430
 Ser Ser Ser Lys Pro Ile Thr Ser Lys Thr Tyr Gln Lys Asn Ser Thr
 435 440 445
 Gly Val Met Ser Thr Ser Thr Asp Val Lys Ser Thr Thr Leu Cys Ser
 450 455 460
 Ser Val Pro Thr Ser Lys Phe Gly Ser Ser Arg Ser Leu Glu Ser Ser
 465 470 475 480
 Val Ile Asp Ser Thr Ser Ser Phe Val Asn Asn Thr Asn His Gln Ser
 485 490 495
 Phe Thr Thr Glu Ser Gly Thr Thr Ser Val Asn Ser Ser Asn Leu Ser

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      500                      505                      510
Thr Gln Ile Ser Arg Met Ser Ser Phe Ser Thr Phe Asp Lys Ala Thr
      515                      520                      525
Thr Lys Ile Ser Ser Ser Val Gln Thr Ser Ser His Ile Leu Gln Ser
      530                      535                      540
Pro Thr Thr Val Ser Tyr Met Asp His Thr Arg Asp Arg Thr Glu Ala
545                      550                      555                      560
Ser Cys Ser Leu Asp Ile Thr Asn Lys Leu Leu Phe Gln Gln Cys Ala
      565                      570                      575
Phe Asn Ser Val Pro Glu Phe Gln Ser Lys Thr Gln Met Ser Arg Ser
      580                      585                      590
Gln Ile Pro Phe Ala Thr Tyr Thr Thr Ser Ser Asp Lys Ser Val Tyr
      595                      600                      605
Tyr Ser Arg Ser Gly Ser Ser Gly Ile Met Val Ser Ile Glu Ser
      610                      615                      620
Ser Ile Gly Phe Ser Ile Ser Lys Thr Asn Ser Glu Asn Thr Lys Asn
625                      630                      635                      640
Pro Gln Ser Ile Ile Leu Gln Val Gln Ser Glu Glu Ala Phe Thr Asn
      645                      650                      655
Tyr Ala Thr Thr Thr Glu Thr Ser Ser Lys Ser Lys Leu Ala Asp Asn
      660                      665                      670
Asn Asp Ser Ala Asn Val Gly Asn Ile Gln Ile Glu Thr Ile Ile Gln
      675                      680                      685
Gly Ser Gly Ser Asp Leu Val Thr Met Thr Gly Ala Thr Val Leu Phe
      690                      695                      700
Phe Phe Ile Val Gln Leu Phe Gly Met Phe
705                      710

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<210> 4

<211> 471

<212> PRT

<213> Candida albicans

<220>

<223> Yak1 functional derivative amino acid sequence

<400> 4

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Ser Ile Asn Ser Ile His Ile Ser Pro Val Asn Ala Leu Ser Ile Tyr
      20                      25                      30
Leu Thr Glu Ser Tyr Ser Leu Cys Gln Pro Arg Lys Phe Gln Tyr Ser
      35                      40                      45
Lys Ser Thr Asn Pro Lys Arg Val Leu Thr Lys Pro Ser Glu Pro Lys
      50                      55                      60
Tyr Asn Asn Gly Tyr Asp Asn Glu Asp Ser Asp Tyr Ile Leu Tyr Val
65                      70                      75                      80
Asn Asp Val Leu Gly Ser Glu Glu Gly Lys Lys Tyr Met Val Leu Asp
      85                      90                      95
Leu Leu Gly Ser Gly Thr Phe Gly Gln Val Val Lys Cys Gln Asn Leu
      100                      105                      110
Asn Asn Gln Thr Val Cys Ala Val Lys Val Ile Lys Ser Lys Pro Ala
      115                      120                      125
Tyr Met Asn Gln Ser Leu Thr Glu Val Arg Leu Leu Glu Phe Leu Asn
      130                      135                      140
Ala Asn Ser Asp Gly Lys Asn Phe Ile Arg Leu Leu Asp Thr Phe Met
145                      150                      155                      160
His Lys Glu His Leu Cys Leu Val Phe Glu Ile Leu Ala Ser Asn Leu

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agtgggtgcat	accaccacga	acaacaaca	cagcacctgc	agcaacattt	atcaccta	180
catcctaata	gccagatat	tttatcatca	tcagattctc	aattcctgtc	acaattaaaa	240
caaaatcaaa	ttcctggatt	ccgtaaccct	tggtttttct	aacaacaaaa	ttctaccctt	300
aatatgttat	catcttcata	atctgcatca	tcttcattat	ctcaatcacc	gactaaatca	360
catcaaatac	cattattaca	acaacatcaa	cctccattac	taaataaaag	attatcattt	420
acaaataaatt	taccaataac	ttatgaacat	gaaagtagta	gtaatagtag	tagtaacaac	480
aacagcagca	gcaatggcaa	tggcaacaat	aataacaatg	ataataatac	taatgtacca	540
ccatatatac	atccaccaac	tcgtgctgat	aatccattta	atcattattt	tgccactcaa	600

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cctaaatata	ataatgggta	tgataatgaa	gatagtga	atattttata	tgttaatgat	1260
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ttcttttaaa	agaaagtcaa	caccaccacc	accaccatca	acaacaataa	taataatact	2040
tcagaaacaa	aaccaattta	tgaacttaaa	tcatttgatg	aatattgtca	attttttagaa	2100
tataaacgac	aaaacaaga	aggtgctaca	tctacaacca	acaacaatac	taatagtagt	2160
agtagtagta	atcatcataa	taataatcat	aaaaaggaac	aaccaaataa	aaattatttt	2220
aaacataaat	tattaaaaga	tattattatt	aattataaat	taccttcaaa	aaaaatgaca	2280
aattcaatga	ttgaaaaaga	atatcatgat	cgattattat	taattgattt	tttaactaaa	2340
gtactcaatt	taaatccatt	agaaagatta	acacctcaag	aagctttaaa	acatccattt	2400
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<210> 6

<211> 2148

<212> DNA

<213> Candida glabrata

<220>

<223> Epa6 nucleic acid sequence

<400> 6

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<210> 7

<211> 2145

<212> DNA

<213> Candida glabrata

<220>

<223> Epa7' nucleic acid sequence

<400> 7

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<210> 8

<211> 2413

<212> DNA

<213> Candida albicans

<220>

<223> Yak1 functional derivative nucleic acid sequence

<400> 8

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ctggaaccta	aataataata	tggtgatgat	aatgaagata	gtgattatat	tttatatggt	240
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ggtacatttg	gacaagtagt	taaagtgtcaa	aatccttaata	atcaaactgt	ttgtgctggt	360
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<210> 9

<211> 809

<212> PRT

<213> Candida albicans

<220>

<221> mutagen

<222> (507)

<223> ALA instead of PHE

<220>

<223> N-MPP1-sensitive mutant Yak1 protein sequence

<400> 9

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20          25          30
Pro Pro Pro Thr Gln Leu Thr Ser Ser Gly Ala Tyr His His Gln Gln
35          40          45
Gln Gln Gln His Leu Gln Gln His Leu Ser Pro Asn His Pro Asn Gly
50          55          60
Gln Asp Ile Leu Ser Ser Ser Asp Ser Gln Phe Leu Ser Gln Leu Lys
65          70          75          80
Gln Asn Gln Ile Pro Gly Phe Arg Asn Pro Trp Phe Ser Gln Gln Gln
85          90          95
Asn Ser Thr Pro Asn Met Leu Ser Ser Ser Ser Ala Ser Ser Ser
100         105         110
Leu Ser Gln Ser Pro Thr Lys Ser His Gln Ile Pro Leu Leu Gln Gln
115         120         125
His Gln Pro Pro Leu Leu Asn Lys Arg Leu Ser Phe Thr Asn Asn Leu
130         135         140
Pro Ile Thr Tyr Glu His Glu Ser Ser Ser Asn Ser Ser Ser Asn Asn
145         150         155         160
Asn Ser Ser Ser Asn Gly Asn Gly Asn Asn Asn Asn Asn Asp Asn Asn
165         170         175         180
Thr Asn Val Pro Pro Tyr Ile His Pro Pro Thr Arg Ala Asp Asn Pro
180         185         190
Phe Asn His Tyr Phe Ala Thr Gln Glu Val Thr Leu Gly Ser Asn Asp
195         200         205
Arg Arg Met Ser Ala Ala Val Asp Gly Thr His Tyr Asn Pro Tyr Gly
210         215         220
Phe Asn Gln Gln Leu Asn Ala Tyr Pro Ser Gly Pro Ala Gly Ile Ala
225         230         235         240
Gly Thr Gln Tyr Leu Asn Asn Pro Asn Leu Gly Pro Leu Val Asn Ala
245         250         255
Asn Arg Arg Ser Ser Val Gly Val Leu Pro Asn Tyr Tyr Arg Gln Gln
260         265         270
Gln Gln Gln Gln Val Gln Asp Gln Ser Thr Ala Ala Ala Ala Ala Ala
275         280         285
Ala Ala Ala Ala Ala Tyr Tyr Leu Pro Pro Ala Arg Leu Gly Arg Ser
290         295         300
Thr Leu Ile Val Gln Tyr Gln Gln Tyr Gln Gln Ala Leu His Asn Gln
305         310         315         320
Arg Ile Thr Lys His Lys Asn Pro Ala Pro Lys Ala Arg Lys Ile Tyr
325         330         335
Asn Lys Leu Asp Leu Thr Pro Lys Phe His Gln His Pro Lys Tyr Arg
340         345         350
Arg Cys Ser Ile Asn Ser Ile His Ile Ser Pro Val Asn Ala Leu Ser
355         360         365
Ile Tyr Leu Thr Glu Ser Tyr Ser Leu Cys Gln Pro Arg Lys Phe Gln
370         375         380
Tyr Ser Lys Ser Thr Asn Pro Lys Arg Val Leu Thr Lys Pro Leu Glu
385         390         395         400

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Pro Lys Tyr Asn Asn Gly Tyr Asp Asn Glu Asp Ser Asp Tyr Ile Leu
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Tyr Val Asn Asp Val Leu Gly Ser Glu Glu Gly Lys Lys Tyr Met Val
      420      425      430
Leu Asp Leu Leu Gly Ser Gly Thr Phe Gly Gln Val Val Lys Cys Gln
      435      440      445
Asn Leu Asn Asn Gln Thr Val Cys Ala Val Lys Val Ile Lys Ser Lys
      450      455      460
Pro Ala Tyr Met Asn Gln Ser Leu Thr Glu Val Arg Leu Leu Glu Phe
465      470      475      480
Leu Asn Ala Asn Ser Asp Gly Lys Asn Phe Ile Arg Leu Leu Asp Thr
      485      490      495
Phe Met His Lys Glu His Leu Cys Leu Val Ala Glu Ile Leu Ala Ser
      500      505      510
Asn Leu Tyr Glu Leu Ile Lys Gln Asn Gln Phe Gln Gly Leu Asn Met
      515      520      525
Lys Leu Val Lys Leu Leu Thr Lys Gln Leu Leu Asp Ser Met Ala Gln
      530      535      540
Leu Lys Asn Phe Gln Met Ile His Cys Asp Leu Lys Pro Glu Asn Ile
545      550      555      560
Leu Leu Cys Gln Pro Asp Lys Pro Asn Ile Lys Val Ile Asp Phe Gly
      565      570      575
Ser Ala Cys Phe Thr Arg Asn Thr Ile Tyr Thr Tyr Ile Gln Ser Arg
      580      585      590
Phe Tyr Arg Ser Pro Glu Val Ile Leu Gly Leu Pro Tyr Thr Glu Ser
      595      600      605
Ile Asp Met Trp Ser Leu Gly Cys Ile Val Gly Glu Leu Phe Leu Gly
      610      615      620
Leu Pro Met Phe Pro Gly Thr Ser Glu Tyr Asn Gln Ile Phe Lys Ile
625      630      635      640
Val Asp Met Leu Gly Pro Pro Pro Arg His Met Ile Glu Val Gly Lys
      645      650      655
Asn Ser Phe Asn Phe Phe Lys Lys Lys Val Asn Thr Thr Thr Thr Thr
      660      665      670
Ile Asn Asn Asn Asn Asn Asn Thr Ser Glu Thr Lys Pro Ile Tyr Glu
      675      680      685
Leu Lys Ser Phe Asp Glu Tyr Cys Gln Phe Leu Glu Tyr Lys Arg Gln
      690      695      700
Lys Gln Glu Gly Ala Thr Ser Thr Thr Asn Asn Asn Thr Asn Ser Ser
705      710      715      720
Ser Ser Ser Asn His His Asn Asn Asn His Lys Lys Glu Gln Pro Asn
      725      730      735
Lys Asn Tyr Phe Lys His Lys Leu Leu Lys Asp Ile Ile Ile Asn Tyr
      740      745      750
Lys Leu Pro Ser Lys Lys Met Thr Asn Ser Met Ile Glu Lys Glu Tyr
      755      760      765
His Asp Arg Leu Leu Leu Ile Asp Phe Leu Thr Lys Val Leu Asn Leu
      770      775      780
Asn Pro Leu Glu Arg Leu Thr Pro Gln Glu Ala Leu Lys His Pro Phe
785      790      795      800
Ile Ile Asp Val Asn Thr Thr Asp Leu
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<210> 10

<211> 778

<212> PRT

<213> Candida glabrata

<220>

<223> Yak1 amino acid sequence

<400> 10

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20          25          30
Asn Ala Asp Ser Lys Arg Phe Ala Phe Thr Asn Pro Trp Gly Asn Glu
35          40          45
Lys Ile Asp Glu Asp Glu Ala Met Ser Tyr Asn Tyr Ser Ser Ala Asn
50          55          60
Ser Thr Ala Asp Val Asp Met Asn Ser Asp Met Tyr Lys Arg Arg Lys
65          70          75          80
Ser Ser Leu Val Val Pro Pro Ser Arg Ala Ala Pro Asn Pro Phe
85          90          95
Gln Tyr Glu Thr Met Gln Asn Ser Thr Gly Thr Ala Gln Arg Ala Met
100         105         110
Tyr Pro Glu Ser Ser Ala Tyr Gln Gln Gln Arg Gln Ile Met Val Pro
115         120         125
His Gln Gln Gln Gly Gln Leu Ala Ser Arg Phe Asn Ser Arg Phe Val
130         135         140
Pro Ser Leu Tyr Asn Pro Gln Glu Phe Gln Arg Arg Gln Ser Leu Ala
145         150         155         160
Thr Ala Gln Phe Ser Thr Pro Ser Ser Leu His Gly Ser Ala Ser Asn
165         170         175
Gln Ala Phe Asn Thr Gly Ser Gly Asn Asn Gly Val Asn Leu Asn Tyr
180         185         190
Ala Ser Ala Asn Val Thr Thr Asn Asn Ser Ser Thr Tyr Leu Ser Ala
195         200         205
Met Thr Pro Asn Val Pro Met Glu Met Gly Asn Ile Pro Leu Ala Ser
210         215         220
Pro His Arg Arg Leu Ser Ala Phe Pro Thr Ser Gly Thr Thr Asn Ser
225         230         235         240
Ser Leu Gln Pro Pro Phe Lys Gln Leu Arg Arg Asp Glu Gly Ser Pro
245         250         255
Arg Gln Ile Val Ile Pro Gln Met His Arg Cys His Ser Lys Asn Asp
260         265         270
Leu Gln Pro Cys Ile Asn Pro Asn Pro Lys Phe Arg Arg Ala Ser Leu
275         280         285
His Ser Asn Thr Ile Ser Pro Leu Ile Gly Leu Thr Lys Ser Leu Leu
290         295         300
Thr Thr Tyr Ala Leu Cys Ser Pro Asp Phe Thr Tyr Gln Thr Ser Lys
305         310         315         320
Asn Pro Lys Arg Val Leu Thr Lys Pro Ser Glu Gly Lys Tyr Asn Asn
325         330         335
Gly Tyr Asp Asn Val Asn Ser Asp Tyr Ile Leu Tyr Val Asn Asp Val
340         345         350
Leu Gly Thr Glu Gln Asn Arg Lys Tyr Leu Val Leu Asp Ile Leu Gly
355         360         365
Gln Gly Thr Phe Gly Gln Val Val Lys Cys Gln Asn Leu Gln Thr Lys
370         375         380
Glu Ile Ile Ala Val Lys Val Val Lys Ser Arg Ser Glu Tyr Leu Asn
385         390         395         400
Gln Ser Ile Ser Glu Ala Lys Ile Leu Glu Leu Leu Asn Glu Lys Ile
405         410         415
Asp Pro Asn Asn Lys His His Phe Leu Arg Met His Asp Ser Phe Val
420         425         430
His Lys Asn His Leu Cys Leu Val Phe Glu Leu Leu Ser Asn Asn Leu
435         440         445
Tyr Glu Leu Leu Lys Gln Asn Lys Phe His Gly Leu Ser Ile Gln Leu
450         455         460
Ile Arg Thr Phe Thr Ile Gln Ile Leu Asp Ser Leu Cys Val Leu Lys

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465		470		475		480
Asp Ser Lys Leu Ile	His Cys Asp Leu Lys Pro	Glu Asn Ile Leu Leu				
	485		490			495
Cys Ser Pro Asp Lys Pro	Asp Leu Lys Val Ile	Asp Phe Gly Ser Ser				
	500		505			510
Cys Glu Glu Thr Arg Thr	Val Tyr Thr Tyr Ile	Gln Ser Arg Phe Tyr				
	515		520			525
Arg Ala Pro Glu Ile Ile	Leu Gly Ile Pro Tyr	Ser Thr Ser Ile Asp				
	530		535			540
Met Trp Ser Leu Gly Cys	Ile Val Ala Glu Leu	Phe Leu Gly Ile Pro				
	545		550			555
Ile Phe Pro Gly Ser Ser	Glu Phe Asn Gln Leu	Thr Arg Ile Ile Thr				
	565		570			575
Thr Leu Gly Tyr Pro Pro	Thr Trp Met Ile Asp	Met Gly Lys Ser Ser				
	580		585			590
His Lys Phe Leu Lys Lys	Leu Asn Thr Leu Asn	Leu Asp Met Thr Asn				
	595		600			605
Ile Asn Asp Val Glu Ala	Gln Asn Asn Asn Ala	Asn Tyr Thr Thr Ala				
	610		615			620
Asn Arg Met Gln Lys Asn	Gln Pro Thr Ala Lys	Tyr Arg Leu Lys Thr				
	625		630			635
Ile Glu Glu Phe Asn Arg	Asp Asn Asn Ala Ser	Glu Lys Val Gly Lys				
	645		650			655
Gln Tyr Phe Lys Trp Ala	Leu Leu Glu Asp Ile	Ile Arg Asn Tyr Arg				
	660		665			670
Ile His Lys Asn Ile Gln	Lys Ser Pro Glu Leu	Ile Glu Gln Glu Met				
	675		680			685
Lys Asn Arg Glu Cys Leu	Ile His Phe Leu Lys	Gly Ile Leu Asn Leu				
	690		695			700
Asn Pro Leu Glu Arg Trp	Thr Pro Gln Gln Ala	Met His Pro Phe				
	705		710			715
Ile Thr Gln Gln Pro Phe	Thr Gly Lys Trp Phe	Pro Pro Gly Ser Phe				
	725		730			735
Asn Thr Met Asn Thr Ser	Asn Phe Gly Ser Gln	Pro Arg Gln Glu Gly				
	740		745			750
Asn Asn Ser Asn Asn Met	Lys Pro Asn Ser Gln	Glu Gly Pro Thr Lys				
	755		760			765
Glu Phe Thr Asn Leu Lys	Ile Ile Glu Asp					
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<210> 11

<211> 2337

<212> DNA

<213> Candida glabrata

<220>

<223> Yak1 nucleic acid sequence

<400> 11

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