Chapter 2
Virulence and Pathogenicity of Fungal Pathogens with Special Reference to *Candida albicans*

Mohd Sajjad Ahmad Khan, Iqbal Ahmad, Farrukh Aqil, Mohd Owais, Mohd Shahid, and Javed Musarrat

Abstract The frequency of severe systemic fungal diseases has increased in the last few decades. The clinical use of broad spectrum antibacterial drugs and immunosuppressive agents after organ transplantation, cancer chemotherapy, and advancements in surgery are associated with increasing risk of fungal infection. Despite the effectiveness of available antifungals in combating such infections, the emergence of drug resistance to antifungals, and problems of toxicity and poor delivery of drugs at the target site in systemic infections, have necessitated a systematic approach to the study of fungal pathogens, host–fungi interactions, and identification of virulence factors. Characterization of virulence factors is expected to improve understanding of fungal pathogenesis and to help explore new drug targets. In this article we discuss the process of fungal infections, virulence factors and pathogenicity of fungal pathogens, with special reference to *Candida albicans*. Adherence, dimorphism, phenotypic switching, secretion of hydrolytic enzymes, biofilm formation,

M.S.A. Khan (✉) and I. Ahmad
Department of Agricultural Microbiology, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh 202002, India
e-mail: msajjadakhan@rediffmail.com

F. Aqil
Brown Cancer Center, University of Louisville, Louisville, KY 40202, USA

M. Owais
Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh 202002, India

M. Shahid
Department of Microbiology, JN Medical College, Aligarh Muslim University, Aligarh 202002, India

J. Musarrat
DNA Research Chair, Department of Zoology, King Saud University, Riyadh, Saudi Arabia

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and ability to adapt at host body temperature are some of the well-known virulence factors among pathogenic fungi and are discussed in relation to *C. albicans*.

2.1 Introduction

Fungi are eukaryotic microorganisms that are more closely related to humans than bacteria at cellular level. They belong to the group Eumycota, and are chemoheterotrophs with a chitinous cell wall. More than 100,000 species have been described. Most species grow as multicellular filaments called hyphae-forming mycelium such as molds; some species also grow as single cells like yeasts. Some groups of fungi are pathogenic to humans and require control measures. Human fungal pathogens belong to four main groups, namely zygomcetes, ascomycetes, deuteromycetes, and basidiomycetes. Fungi can cause significant number of human diseases represented by pathogens such as *Trichophyton* sp, *Epidermophyton* sp, *Histoplasma* sp, *Blastomyces* sp, *Sporothrix* sp, *Coccidioides* sp, and *Paracoccidioides* sp, capable of infecting healthy people, or opportunistic invaders such as *Aspergillus* sp, *Candida* sp, *Cryptococcus* sp, *Fusarium* sp, and *Rhizopus* sp, which are normally avirulent in healthy people but could be disseminated to deep tissue and cause fatal disease in unhealthy people (Chakrabarti 2005; Reedy et al. 2007). The morbidity and mortality rates caused by fungal species such as *Candida, Aspergillus, Fusarium, and Trichosporum* are relatively higher (Fluckiger et al. 2006). In Europe, fungal infections account for 17% cases associated with intensive care units (Rupp 2007), while in the USA it has become the seventh most common cause of deaths among hospitalized patients (Martin et al. 2003). About 15% of allogenic haemopoietic stem cell transplant recipients and 20% of lung transplant recipients suffered fungal infections (Ribaud et al. 1999). Approximately 60% and 20% of AIDS patients present with pneumonia and esophageal candidiasis respectively (Moore and Chaisson 1996).

Data from the late 1950s and early 1960s indicate that invasive fungal infections were extremely rare, even in immunocompromised cancer patients (Chakrabarti 2005). Now, fungal infections have dramatically increased in the past two decades as a result of improved diagnostics, high frequency of catheterization, instrumentation and an increasing number of immunosuppressed patients. Particularly, invasive fungal infections are showing extremely high mortality rate. The use of antineoplastic and immunosuppressive agents, broad-spectrum antibiotics, prosthetic devices and grafts, and more aggressive surgery have led to the development of complicated infections, including invasive fungal infections. Furthermore, patients with burns, neutropenia, and HIV infections are now seriously exposed to fungal infections (Kuleta et al. 2009).

Fungal infections have now also become more common in the healthy population. The National Nosocomial Infections Surveillance System has reported *Candida* spp. as the fourth most common bloodstream isolates in nosocomial infections in USA. Over 95% of all fungal infections have been associated with *Candida albicans, Aspergillus fumigates*, and *Cryptococcus neoformans* (Richardson 2005).

2.2 Diseases Caused by Human Pathogenic Fungi

Fungal diseases can be broadly classified on the basis of causative agents as: (a) dermatophytosis, (b) histoplasmosis, (c) blastomycosis, (d) coccidiomycosis, (e) candidiasis, (f) cryptococcosis, (g) aspergillosis, (h) hyalohyphomycosis, and (i) zygomycosis, as described by many authors (Sullivan et al. 2005). These diseases differ in their nature, causative agents, and distribution. Description of such fungal diseases, their causative agents and major organs involved etc are given in Table 2.1. However, candidiasis is described here briefly. Candidiasis encompasses secondary or opportunistic infections ranging from acute, sub-acute, and chronic to life-threatening mycoses. Infections are localized to mouth, throat, skin, vagina, fingers, bronchi, lungs, and gastrointestinal tract, or sometimes become

Table 2.1 Examples of commonly caused fungal diseases

Fungal diseases	Causative agent	Site of infection	Transmission
Dermatophytosis	*T. rubrum, T. mentagrophytes, E. floccosusm, M. gypseum, M. canis*	Skin, hair, nails, feet	Soil, contact with arthrospores or conidia from contaminated animals and humans
Histoplasmosis	*H. capsulatum*	Lungs	Soil, inhalation of microconidia
Blastomycosis	*B. dermatididis*	Lungs, skin, genitourinary tract, brain	Soil, inhalation of conidia
Coccidioidomycosis	*C. immitis, C. posadasii*	Lungs, bones, joints, meninges	Soil, inhalation of arthroconidia
Candidiasis	*C. albicans, C. tropicalis, C. glabrata, C. dubliniensis, C. krusei*	Intestinal tract, vaginal tract, skin, fingers, oral cavity	Endogenous flora, contact with secretions from infected person
Cryptococcosis	*C. neoformans*	Lungs, meninges, kidney, liver, prostate, bones	Soil, contamination with bird feces
Aspergillosis	*A. fumigatus, A. flavus, A. niger*	Lungs	Soil, inhalation of spores
Hyalohyphomycosis	*Fusarium* sp., *Phecilomyces* sp, *Scedosporium* sp, *Scopulariopsis* sp, and *Acremonium* sp	Keratin, nails, lungs	Soil, plant debris, ingestion of toxin contaminated plant parts
Zygomycosis	*Rhizopus* sp, *Mucor* sp and *Absidia* sp	Skin, cerebral, blood, lungs, genitourinary and gastrointestinal system	Soil, decaying plant material, inhalation or percutaneous contact of spores

Adapted from Weitzman and Summerbell (1995), Hogan et al. (1996), Pommerville (2004), Rappleye and Goldman (2006), Willey et al. (2008)

systemic as candidemia, endocarditis, and meningitis. A number of *Candida* spp are encountered in candidiasis such as *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. dubliniensis, C. parapsilosis* (Hayens and Westerneng 1996). *C. albicans* is a member of the commensal microflora of the intestine. It is pleomorphic and undergoes reversible morphogenic transitions between budding yeast, pseudohyphal, and hyphal growth forms. Healthy persons generally encounter superficial infections but in immunocompromised patients invasive infections could also occur. Approximately 70% of woman experience vaginal candidiasis once in a life, and 20% suffer from recurrence (Fidel et al. 1999). Among other *Candida* spp, *C. glabrata* has emerged as a frequent pathogen due to increased use of immune suppressive agents. *C. krusei* is a pathogen of importance in patients with hematological malignancies and transplants. *C. parapsilosis* is frequently isolated from blood cultures due to insertive medical devices. *C. tropicalis* is one of the causative agents of candidemia and isolated from patients with leukemia and those who have undergone bone-marrow transplantation. *C. dubliniensis* is found associated with systemic infections in AIDS patients.

2.3 Host–Fungi Interaction: The Process of Infection

Like any other microbial pathogen, fungal infection also involves some basic steps such as (1) entry and adherence to the host tissue, (2) invasion of the host tissue, (3) multiplication, colonization and dissemination in the tissues, and (4) evasion of the host immune system and damage to the tissues.

2.3.1 Entry or Adherence to the Host Tissue

Humans are first exposed to fungus *C. albicans* when passing through the vaginal canal during birth. In this course the fungus colonizes the buccal cavity, and upper and lower parts of the gastrointestinal tract of the newborn, where it becomes commensal (Khan and Gyanchandani 1998; Cladernone and Fonzi 2001). Other fungi of human diseases come from exogenous sources of soil and decaying vegetation as saprophytes. Generally, they enter through respiratory portals. Fungi rarely cause disease in immunocompetent hosts, though often exposed to infectious spores. Disease results when fungi accidentally penetrate host barriers or when immunologic defects or other debilitating conditions exist that favor fungal entry and growth (Hogan et al. 1996). Infection of a host starts with the adherence of fungi at epithelial surface layers and further dissemination to different host sites. Invasion of various tissues and resistance to attack by the host immune system is necessary for a pathogen to establish infection.

2.3.2 Adaptation and Propagation

For a fungus to survive in its niche it has to adapt to constantly changing parameters. Therefore, fungi respond to change in a specific environmental component by inducing transcriptional and translational changes that promote survival under the newest environmental conditions. When fungi enter the mammalian host their lifestyle changes from saprophytic to parasitic. As saprophytes, fungi survive in an environment with a moderate ambient temperature and pH, essential nutrients such as carbon and metal ions, and atmospheric concentrations of carbon dioxide and oxygen. Once having invaded a human host, these environmental factors are suddenly replaced by drastic changes. In the different niches of a host, completely different nutrient compositions may exist and specialized features of fungal pathogens may be involved in the establishment, dissemination, and manifestation of an infection (Brock 2009). For example, ambient temperature is replaced by the high temperature of the human body. Fungal survival at the elevated temperature of a human host is essential for virulence. The fungal pathogens *C. neoformans* and *A. fumigatus* are simply better able to survive at 37°C than their nonpathogenic counterparts (Hogan et al. 1996). Fungi often develop morphogenetic virulence mechanisms, e.g., formation of yeasts, hyphae, and spherules that facilitate their multiplication within the host at higher temperature. Yeast cells of many *Candida* species form filamentous pseudohyphae and hyphae in tissues, whereas *C. neoformans* yeasts become coated with a capsule, and *Coccidioides immitis* develops swollen, septated spherules in the host. Other fungi such as *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *Penicillium marneffei* form filamentous mycelia in the environment, but convert to yeast morphology upon contact with the human host (Rappleye and Goldman 2006). Hyphae that grow in the skin or nail as dermatophytes can fragment into arthroconidia or other conidial types. On the other hand, ambient pH is replaced with acidic conditions of mucosal surfaces or neutral to slightly alkaline pH of blood and tissues. One pathway used by fungi in response to changing pH involves activation of the transcription factors such as PacC in *A. nidulans* and Rim101 in *C. albicans* (De Bernardis et al. 1998). Carbon and metal ions are lacking in host tissues; iron is sequestered from microbes by iron carrier proteins in tissues, creating an iron-limited environment. In order to survive, fungi encode certain mechanisms by employing siderophores, high affinity iron chelators, to efficiently bind host iron into fungal cytoplasm (Haas et al. 2008). Also, fungi have to face hypoxia and high levels of carbon dioxide in tissues. In *C. albicans*, the response to hypoxia is dependent on coordination of specific transcriptional regulators; for example, transcription factor Ace2 represses oxidative metabolic processes and promotes filamentation (Mulhern et al. 2006).

All these specialized adaptations help fungi in sustaining infection at the host site. Most of the free-living pathogenic fungi possess an extremely versatile metabolism which allows them to adapt immediately to changes in the environmental conditions during life in the soil. Therefore, success of infection depends on rapid adaptation to changing micro-environments.

2.3.3 Dissemination

Dissemination of fungi in the host body is facilitated by severe endocrinopathies and immune disorders. A fungus utilizes various mechanisms to deceive or destroy the immune cells and spread to various organs. Dissemination depends on interactions of factors from host and fungi, as described by several authors (Casadevall and Pirofski 2001; Latge and Calderone 2002).

2.3.3.1 Host Factors

Considering the interaction between host and pathogen, immune cells are the major antagonists to the survival of fungal pathogens inside the host. However, primary resistance to fungal invasion and colonization is contributed by cutaneous and mucosal physical barriers. The non-specific host defenses include (1) the antifungal activity of saliva and sweat, (2) the competition for space and nutrients by the normal microbiota of the skin and mucous membranes, which limits the growth of potential pathogens, and (3) the mechanical barrier of the skin and mucous membranes which prevent entry of fungi. Inflammatory systems to combat fungal proliferation involving the action of neutrophils, mononuclear phagocytes, and other granulocytes are also considered to be nonspecific. The specific host defenses or acquired immunity consist basically of the cell-mediated immunity regulated by T-lymphocytes. In humans, mycoses acquired by exposure to fungal spores through the respiratory tract are checked primarily by the first line of defense, i.e., mucociliary clearance. Remaining spores are ingested and killed by monocytes or macrophages through phagocytosis as adaptive innate immunity (Wanner et al. 1996). In addition, healthy individuals employ a second line of defense formed by neutrophilic granulocytes. They mainly attack hyphae, which are too large for ingestion. These in turn are killed by oxidative and non-oxidative mechanisms, including different defensins. Each of these two defense systems alone is able to protect the host against large spores over long time periods. Fungal pathogens can cause invasive disease only if both protective lines are surpassed (Murphy 1991). Overall, severity of disease depends on factors such as inoculum size of the attacking pathogen, magnitude of tissue damages, ability of fungi to multiply in the tissue, and the immune status of the host cells.

2.3.3.2 Fungal Factors

Production of extracellular enzymes such as keratinases, collagenases, gelatinases, phospholipases, lipases, and acid proteinases by dermatophytes, *Aspergillus* sp, *Candida* sp, and *Cryptococcus* sp is considered to be the fungal-associated factor that helps fungi in nutrient uptake, tissue invasion, adherence, and dissemination inside the host. In some fungi such as *C. neoformans*, the presence of capsule may be

an important factor. Similarly, the ability to grow at 37°C, dimorphism, and other factors contribute to fungal pathogenesis, which involves a complex interplay of many fungal and host factors.

2.4 Virulence and Pathogenicity

Pathogenesis is the ability of a microorganism to infect the host and produce disease resulting from interaction of pathogen with host via expression of certain factors on both sides. Pathogenicity of a fungus depends on its ability to adapt to the tissue environment and to withstand the lytic activity of the host's defenses. Several determinants including genes or gene products such as enzyme molecules known as virulence factors are involved in this relationship, producing superficial to invasive infections in humans. Virulence refers specifically to a property of the pathogen and, according to modern definitions, virulence is the ability of a pathogen to multiply and cause harm to its host (Casadevall 2007). For a fungus to produce disease in a patient, it must be actively invading tissues. Diseases caused by fungi without invasion of live tissues include mould allergies and cutaneous dermatophyte infections (ringworm), in which fungi invade and damage only the nonviable epidermis. Further, potentially lethal mycoses involving deep tissues result from fungal dissemination and invasion throughout the body (Fluckiger et al. 2006). Many human fungal pathogens are dimorphic (capable of reversible transitions between yeast and hyphal forms), and the morphogenetic transition between these forms is often stimulated by growth in the host and correlated with host invasion. However, the nature of association between fungal morphogenesis and host invasion is a highly debated aspect of fungal virulence (Molero et al. 1998; Klein and Tebbets 2007).

Determinants of pathogenicity are called virulence factors. Pathogenic microbes often possess a number of virulence factors and mechanisms. These factors determine whether the organism (the host) lives or dies during host–microbe interactions. The factors can be inducible or constitutive, the direct product of genetic elements (proteins), or the products of complex biosynthetic pathways such as polysaccharides or lipid mediators. The virulence factor can be assessed by comparing biological response in fungi with and without the factor. The most convincing evidence for a factor to be considered as a virulence determinant is the simultaneous loss of the factor and loss of virulence, and the regaining of virulence when the factor is restored. Virulence factors must help the pathogen to grow at elevated temperatures, facilitate adherence, penetration and dissemination, or assist in resistance against innate immune defenses, e.g., phagocytosis and complement, evasion from adaptive immune defenses, or nutritional and metabolic factors, necrotic factors, or morphology variation. The ability of a fungus to grow at 37°C and physiological pH is a virulence factor for fungi that invade deep tissue, and the transition to parasitic form is essential for the pathogenicity of dimorphic fungi. A size compatible with alveolar deposition is a virulence factor for fungi producing

infections by inhalation of airborne spores (Tomee and Kauffman 2000). Some kinds of virulence factors are commonly required for all pathogens, such as the ability to recognize and adhere to host tissues, to respond rapidly to changes in the external environment and to secrete hydrolases; all are thought to be important in virulence. But the complex nature of the host–fungus interaction has resulted in some factors that are absolutely required for fungal virulence. Some properties are frequently associated with pathogenesis across all fungal pathogens and others have been found to be important for specific pathogens. Because pathogenesis is complex phenomenon, possession of a single putative virulence factor is not sufficient for a fungus to become pathogenic; rather, a complex combination of properties is usually required. Several kinds of processes are thought to be involved in virulence in a wide range of fungal pathogens. Virulence factors associated with certain well-characterized fungi have been described in the literature, and we have listed some of them associated with medically important fungi in Table 2.2.

2.5 *Candida albicans*: An Opportunistic Fungus

Candida spp are asexual yeasts of the genus ascomycetes and genetically diploid with the presence of eight chromosomes. Some species have shown phenotypic switching, variant colony morphology and dimorphism, and transition from yeast to filamentous form. Out of more than 200 species, the most commonly encountered in medical practices are *C. albicans, C. dubliniensis, C. glabrata, C. krusei, C. parapsilosis,* and *C. tropicalis*. About 8%–15% of nosocomial blood stream infections are reported to be caused by *Candida* spp (Pfaller and Diekema 2002). Candidal infections are a serious problem in individuals with weakened immune defense. Interestingly, *C. albicans* differs from other medically important fungi such as *H. capsulatum, A. fumigatus,* and *C. neoformans* in rarely being isolated from soil. Therefore, infections caused are categorized as endogenous and not exogenous as with others. *C. albicans* and related spp have been isolated from several body locations as a carrier in the oral cavities, gastrointestinal tract, anus, groin, vaginal canal, and vulva of healthy people, and may attain sufficiently high density without symptoms of disease. Among these, *C. albicans* was predominant at all body locations (70%), *C. glabrata* and *C. tropicalis* (7%) (Odds 1988). In normal conditions, it exists with other normal microbial flora of host organs; about 50% of a healthy population is supposed to be a benign carrier of *Candida* spp, but in immunocompromised patients who have undergone chemotherapy, bone-marrow transplantations, or diabetic treatment, it behaves like an opportunistic pathogen and produces superficial to systemic infection. Broad-spectrum antibiotic therapy may also alter the population of normal bacterial flora, resulting in *Candida* sp taking over the niche and assisting in flourishing and establishing secondary infections. Oral and vaginal thrushes are very common even in individuals with slightly weakened immunity (Soll 2002a; Fluckiger et al. 2006; Odds et al. 2006). The ability of *C. albicans* and other *Candida* spp to colonize and survive at different

Table 2.2 Role of virulence factors in pathogenic fungi

Fungal pathogen	Type of virulence factors	Role in pathogenicity
Dermatophytes	Keratinase	Damage of keratinous layer in epidermis
	Elastase	Destruction of elastin in tissues
	Acid proteinase	Cleavage of peptide bonds in host cells to obtain nutrients and invasion
	Phospholipase	Cleavage of phosphodiester bond in membrane lipids for invasion
Aspergillus spp	Cell wall component, β-1,3 glucan	Cell adhesion
	Conidial size (2–3 μ)	Escape from mucocilliary extrusion
	Adhesin, tronchin	Binding of conidia to lung tissue
	cAMP, rasA, rasB	Nutritional uptake and growth of pathogen, germination of conidia, branching of hyphae
	Elastase-alkaline serine proteinase	Degradation of elastin in lung tissue
	Phospholipase C (plb1,2,3)	Tissue damage and penetration
	Catalases (catA, catB and cat2), Superoxide dismutase	Prevention from oxidative damage in macrophages
	Gliotoxin, helvolic acid	Immunosuppressive properties, prevention from oxidative burst of macrophages
	Ribotoxin	Cleavage of phosphodiester bond in eukaryotic 28s rRNA
	Siderophore (sidA gene)	Uptake of iron from blood heme
	Growth at 37°C, hsp1, cgrA	Ability to invade host tissue and survival at elevated temperature of host
Histoplasma capsulatum	Dimorphism	Altered cell surface adhesion, tissue invasion by hyphal phase, dissemination by yeast phase
	α-1,3-glucan in cell wall	Required for adhesion
	Growth inside macrophage	Evasion from immune cells, dissemination to other organ tissues
	Catalase	Protection from oxidative killing
Coccidiodes immitis	Dimorphism	Sheer size of spherule is required for dissemination, hyphal phase tolerate pH 2–12
	Elastase	Destruction of lung insertium and blood vessels
	Estrogen binding protein	Acceleration of spherule maturation and endospore release
Blastomyces dermatitidis	Dimorphism	Tissue invasion and dissemination
	Adhesin (BAD1)	Suppression of immune response
	α-1,3-glucan in cell wall	Adhesion and masking of cell surface receptors being recognized by immune cells
Cryptococcus neoformans	Capsule	Inhibition to phagocytosis
	Melanin	Prevention from oxidative damages
	Mannitol	Scavenging of hydroxyl radical during respiratory burst
	Phospholipases A,B,C,D (plb1,2,3)	Tissue invasion and adherence
	Acid proteinases	Tissue invasion and dissemination
Candida albicans	Adhesin (Als family, HWP1)	Adherence to epithelial cells, fibronectin, biofilm establishment

(*continued*)

Table 2.2 (continued)

Fungal pathogen	Type of virulence factors	Role in pathogenicity
	Dimorphism (phr1, hyr1, chs2, chs3, rbf1)	Hyphal phase required for invasion and adherence, yeast phase for dissemination
	Phenotypic switching (efg1)	Conversion to more virulent forms showing increased ASP and adhesion production, evasion from host response
	Secreted aspartyl proteinases (SAPs 1–10)	Nutrient uptake, tissue invasion, adherence and dissemination
	Phosphlipases A,B,C,D (plb1,2,3)	Tissue invasion and adherence
	Farnesol	Quorum sensing, Biofilm formation
	Catalase, Superoxide dismutase	Prevention from oxidative damages
	SUN41, GCN4, MKc1p	Biofilm establishment

Adapted from Hogan et al. (1996), Ghannoum 2000, Cladderone and Fonzi (2001), Yang (2003), Blankenship and Mitchell (2006), Kuleta et al. (2009)

anatomic sites of the host has made them more harmful than other commensals of the human body.

Microorganisms colonizing the gut can normally cause dysfunctions of intestine such as *E. coli* or *Salmonella* sp, but opportunistically *Candida* cells can disseminate from gut to oral, vaginal mucosa, or skin as superficial infections and to the bloodstream as systemic infections. *Candida* makes this spread possible by expressing tissue site-specific metabolites. For example, in host tissue or bloodstream having neutral or slightly alkaline pH, it expresses the gene PHR1 whose products are involved in cell wall synthesis and work at neutral pH. On the other hand, in vaginal mucosa where pH is acidic, this gene is switched off and another gene, PHR2, is expressed with similar functions (Saporito-Irwin et al. 1995; Muhlschlegel and Fonzi 1997).

As a commensal, *Candida* resides in yeast form and multiplies by budding into blastospores, but during weakened immunity of the host it transforms into the hyphal form as the start of pathogenesis. In vivo study has revealed that change in pH, oxygen, carbon dioxide, or glucose concentration in host tissue triggers this transition (Cladderone and Fonzi 2001; Haynes 2001). Filamentous forms are more adhesive due to increased expression of adhesins on the surface, and also secretion of a higher amount of hydrolytic enzyme enhances the invasiveness. Moreover, the pathogenic stage has to resist recognition by the immune system or damaging macrophages and neutrophils. This interaction with host tissue in favor of *Candida* results in deep tissue penetration and the establishment of infections. It is speculated that host (tissue environment and immune system) alone determines the balance between commensalism and pathogenicity (Soll 2002a; Hube 2004).

The work of several researchers has shown that certain genes such as pH-regulated PHR1 and PHR2, genes encoding secreted aspartyl proteinases (SAPs 1–9), and genes encoding phospholipases (PHL A–D) are expressed differentially in specific tissues at different stages of infections (Yang 2003; Naglik et al. 2004).

Expression or modulation of these genes on the same mucosal surfaces only during transition from the commensal to the parasitic stage reflects a weakness in the immune system responsible for this shift (Casadevall and Pirofski 2001). *Candida* survives and proliferates as commensal in competition with other microbial flora and is affected by epithelial cell proliferation and the immune system. Proliferation of epithelial cells constitutively forces *Candida* to attempt deeper invasion into tissues. Prolonged antibiotic therapy provides more available nutrients and space for *Candida* to multiply as other commensal microbial flora are diminished (Senet 1998). Immune suppression in HIV patients and inhibition of epithelial cell proliferation such as in cancer therapy changes the tissue environment in terms of pH, osmolarity, and oxidative stress. This changed condition is perceived by the candidal cell and subsequently down- or up-regulation of certain genes provokes *Candida* to switch over from commensal to opportunistic pathogens (Claderone and Fonzi 2001; Hube 2004).

Advanced medical equipment and surgery has also led to the increased spread of commensal *Candida* to tissues as pathogens. Medical devices such as catheter, dental implants, artificial joints, pacemakers, central nervous system shunts, and others have provided the opportunity to form biofilms, a stage more resistant to drugs and capable of greater invasion to tissues. These devices are easily colonized by candidal cells from mucosal surfaces and blood stream, and frequently get spread from one tissue site to another. Further, candidal cells can also migrate via blood flow to all inner organs, leading to septicemia and life-threatening diseases (Douglas 2003; Hall-Stoodley et al. 2004). Biofilm-forming cells have been reported to be more virulent than planktonic cells (Ramage et al. 2005; Seneviratne et al. 2007). Recently, several workers have reported increased production of proteinases, phospholipases, and adhesins in biofilm compared to planktonic cells (Chandra et al. 2001; Al-Fattani and Douglas 2006; Seneviratne et al. 2007). All these collectively aid in establishing infections by heightening the adherence and invasion of tissues, leading to increased virulence. Further, genetic changes in biofilms result in elevated drug resistance, pronounced quorum sensing and regulated carbohydrate synthesis, thereby influencing the pathogenicity of *Candida*. Therefore, biofilms-forming capacity has greatly increased the potency of *Candida* to convert from the commensal stage into a virulent pathogen.

2.6 Virulence Factors Involved in Pathogenicity of *Candida albicans*

Like other fungal pathogens, *C. albicans* also regulates expression of certain genes and their products as virulence factors to produce disease. This is the most common opportunistic pathogen, utilizing several kinds of virulence factors. Some of the commonly studied virulence factors in *C. albicans* are briefly described here.

2.6.1 Adhesion

Adherence of candidal cells to host tissues is a complex multifactorial phenomenon utilizing several types of adhesins expressed on morphogenetically changing cell surfaces. But the striking feature of *Candida* cells is the formation of biofilms in host tissue, resulting in enhanced adherence. Ramage et al. (2006) have reported that in the last few decades, *Candida*-related infections have been found associated with biofilm-forming capacity. Well-known adhesins are agglutinin-like sequences (ALS) that are members of a family of seven glycosylated proteins. Als1p, Als3p and Als5p (Ala1p) on the cell surface of hyphae adhere to human buccal epithelial cells (HBEC) and fibronectin, collagen, laminin, and endothelial cells (Hawser and Douglas 1994; Hoyer 2001). Als6p and Als9p bind to collagen and laminin respectively. Als4p binds to endothelial cells and Als5p is additionally needed for cell aggregation. However, the role of Als7p is unclear (Filler et al. 2006; Kuleta et al. 2009). Another 34 kDa adhesin molecule, Hwp1 (hyphal wall protein), encodes an outer surface mannoprotein on the hyphal wall; the amino terminal sequences of this adhesin are recognized as mammalian transglutaminase substrate (TGase) and form covalent binding with HBEC. Studies with hwp1$^-$ knockout mutant and HWP1$^-$ deficient mutant of *C. albicans* have shown reduced adherences and mortality in murine models (Chaffin et al. 1998; Staab et al. 1999). An integrin-like protein (Int1p) which is a plasma membrane receptor and antigenic functionally similar to human complement receptors 3 and 4, has been isolated from *C. albicans* and found to bind with extracellular matrix (ECM) ligands such as fibronectin, laminin, and collagen I and IV, and induce morphological changes in response to extracellular signals (Cladera one and Fonzi 2001; Ruiz-Herrera et al. 2006).

2.6.2 Morphogenesis

Morphogenesis in *C. albicans* is defined as transition from unicellular yeast form to filamentous form (pseudohyphae or hyphae). Of all the species only *C. albicans* and *C. dubliniensis* are able to undergo morphogenesis. Transition from yeast form to hyphal form is facilitated by nutrients, near-neutral pH, temperature of 37°C–40°C, CO_2 concentration about 5.5%, and presence of *N*-acetyl-D-glucosamine, serum, some amino acids, and biotin. Reverse transition from hyphal to yeast form is provoked by lower temperatures, acidic pH, absence of serum, and higher concentration of glucose (Corner and Magee 1997; Eckert et al. 2007). This transition is strongly required for pathogenesis. Yeast forms are more suited for dissemination in tissues and to other hosts, whereas hyphal forms are required for tissue damage and invasion. For example, the yeast cell, when phagocytosed by macrophages, produces hyphae and secretes hyphae-associated proteinases that kill macrophages; these factors also prevent hyphal cells from being killed by neutrophils. In addition, hyphal cells have been shown to induce phagocytosis by endothelial cells, helping

Candida cells to escape from the bloodstream (Molero et al. 1998; Gow et al. 2002; Hube 2004). Further, hyphal cells have stronger adherence capacity due to expression of ALS adhesins and also exhibit greater invasiveness to tissues. Increased expression of superoxide dismutase (SOD) antagonizes oxidative burst of phagocytic cells. Several genes have been identified which regulate phase transition, namely PHR1, ECE1, HYR1, RBF1, CHS2, CHS3 which are differentially expressed during morphogenesis (Haynes 2001; Claderone and Fonzi 2001). Of these, ECE1 correlates with hyphal elongation although ECE1 null mutants displayed no morphological alterations. Similarly, null mutants for expression of CHS2, CHS3 and HYR1 did not show any obvious morphological type. But disruption of RBF1 demonstrated alteration in cell morphology and strongly involved in yeast–hypha transition (Yang 2003). Studies with homozygous null mutants for Hst7p, Cph1p and Cst20p have shown defective hyphal formation (Leberer et al. 1996); in addition, three genes *TUP1*, *EFG1*, *CLA4* were found to be regulating candidal morphogenesis (Liu 2001). Transcription factors such as Tup1 and Rbp1 are negative regulators of filamentation (Braun and Johnson 2000). A tup1 mutant strain resulted in constitutive filamentous growth under all conditions, indicating a role in filament formation. Deletion of homozygous allele of Ste20 encoded by CLA4 showed impaired hyphal formation in a wide range of medium, and decrease in virulence in a murine model (Braun and Johnson 1997; Celera and Claderone 2001). A protein of bHLH class encoded by EFG1 acts as transcriptional activator as well as repressor, and is required for pseudohyphal and hyphal morphogenesis (Liu 2001; Noffiz et al. 2008). A study with homozygous mutants cfg1 and cph1 showed failure of germ tube and hyphae production in a murine model (Noffiz et al. 2008).

2.6.3 Phenotypic Switching

Unlike other pathogens, phenotypic switching in *Candida* is pleitropic by affecting several phenotypic and metabolic parameters, with subsequently a number of virulence traits such as SAP gene regulation. This allows *Candida* to adapt to a different host environment during infection (Soll 1992; Soll 2002b). Colonies of *C. albicans* show morphological variation, including smooth, rough, star, stippled, hat, wrinkle, and fuzzy at high frequency. This switching is reversible, occurs spontaneously in stress, and results in changes in cell surface behavior, colony appearance, and metabolic, biochemical and molecular attributes to become more virulent and effective during infection (Soll 2002b; Odds et al. 2006). Strains isolated from vaginitis or systemically infected patients showed higher frequencies of switching, indicating a strong role for the switching phenomenon in establishing diseases (Kvaal et al. 1999). In the case of yeast–hypha transition, all cells of a population express the same phenotype under the same environmental conditions, whereas in the case of switching, some cells of a population express different phenotypes under the same set of environmental conditions. Earlier research had

reported that laboratory isolate 3153A, grown on amino acid rich agar which was limiting for zinc and incubated at 25°C, showed a smooth phenotype as dominant, while variant colonies of star, ring, irregular, and wrinkle occurred spontaneously. Such types of variation were also observed with cells of strain 3153A treated with low doses of UV irradiation (Soll 1992). At present, of all the phenotypes described, the white-opaque system in strain WO-1 is the most studied. This system is characterized by transition from smooth, white colonies to flat, gray opaque colonies. White cells are round ovoid while opaque cells are elongated or bean shaped (Soll 2002b). Study of gene expression with the WO-1 system revealed an association of OPA1 (SAP1) and SAP3 in opaque cells, in contrast to SAP2, WH11and EFG1 in white cells (Soll 1997; Miller and Johnson 2002). Study with efg1 null mutants exhibited no involvement of EFG1 in switching, but rather control of phenotypic characteristics. It has been reported that white cells in WO-1 hardly form hyphal stages, but this was achieved by opaque cells (Staib et al. 2002). There is good evidence that opaque cells are more virulent than white cells in several murine models (Yang 2003).

2.6.4 Phospholipases

Phospholipases are enzymes that hydrolyze ester linkages of glycophospholipids and hence impart tissue invasiveness to *Candida* cells. In *C. albicans*, four types of phospholipases are classified by researchers on the basis of the ester bond they cleaved, viz., phospholipase A, B, C, and D. All types possess hydrolase activity, but PLB in addition also possesses lysophospholipase transacylase activities; therefore, it is able to release fatty acids from phospholipids and the remaining fatty acid from lysophospholipids, and then transfer a free fatty acid to lysophospholipids, producing phospholipids. Of these, only PLB1, a 84 kDa glycoprotein isolated from hyphal tip in the course of tissue invasion, has been shown to be required for virulence in a murine model of candidiasis (Ghannoum 2000; Yang 2003; Theiss et al. 2006). A study conducted by Ibrahim et al. (1995) revealed an increased level of phospholipase production in blood isolates compared to commensal isolates.

2.6.5 Proteinases

Secretion of proteinases by pathogen is mandatory in order to degrade the tissue barriers and obtain nutrition at the infection site. Secreted aspartyl proteinases (SAPs) from *Candida* have been reported that hydrolyze many proteins such as albumin, hemoglobin, keratin, collagen, laminin, fibronectin, mucin, salivary lactoferin, interleukin1β, cystatin A, and Immunoglobulin A (Hube et al. 1998). To date, ten proteins have been recognized as SAP family (SAPs 1–10) and found to be responsible for tissue invasion. Several researchers have reported that production of SAPs is also correlated with hyphal formation, adherence, and phenotypic switching

(Monod and Zepelin 2002; Naglik et al. 2003). Such researches have highlighted the complex role played by SAPs in the pathogenicity of *C. albicans*. Several models using SAP inhibitors such as pepstatin A and SAP-disrupted or over-expressing mutants demonstrated the need for these factors in candidal pathogenesis. In vitro studies have reported that SAPs 1, 2 and 3 are expressed by the yeast phase, only while SAPs 4, 5 and 6 are expressed in the hyphal phase (Hube et al. 1998; Schaller et al. 1999; Naglik et al. 2004). Whereas, SAPs 9 and 10 are expressed by both forms (Albrecht et al. 2006). Structural analysis revealed that SAPs 1–8 are secreted extracellularly, but that SAPs 9 and 10 are anchored to the cell wall by glycophosphotidyinositol (GPI) protein (Naglik et al. 2003; Albrecht et al. 2006). Models of epidermal and vaginitis candidiasis revealed involvement of SAPs 4–6 in invasive systemic disease whereas SAP 7 was never detected *in vitro*. The role of SAPs 1–3 is associated with early adherence, invasion, and cutaneous infections as studied in the WO1 strain, whereas SAP8 is associated with extensive penetration. SAPs 6 and 9 were found expressed in later stages of hyphal growth (Hube et al. 1998; Schaller et al. 1999; Kvaal et al. 1999). Different properties of SAPs are exploited in the pathogenicity of *Candida*. For example, SAPs are active across a broad range of pH 2.0–7.0, as SAPs 1–3 are active at pH 3.5, SAPs 4–6 at pH 5.0–7.0, and therefore make *Candida* capable of colonizing and invading different tissue sites of varying pH. In addition, SAPs show varied levels of protein specificity, as SAPs 1, 2, 3 and 6 cleave peptide bonds in larger hydrophobic amino acids; SAPs 1, 2 and 6 act on phenylalinine, whereas SAP 3 attacks leucine and SAPs 9 and 10 hydrolyze yapsin and kexins (Naglik et al. 2004). This attribute enables *Candida* to obtain nitrogen at different tissue makeups, and aids pathogenicity by revealing potential binding sites from tissue for adhesion of candidal cells, and also dissemination via circulatory systems. In vivo studies have confirmed the role of SAPs in colonization, increased adhesion and tissue penetration (Naglik et al. 2004; Hube and Naglik 2001). Disruption of SAPs 1, 2 and 3 have resulted in decreased virulence in mouse models (Hube et al. 1997). Several reports have supported functional role of SAP2 in invasion and dissemination of systemic infections (Kvaal et al. 1999; Naglik et al. 2004; De Bernardis et al. 1999). Further research data have indicated increased expression of SAP genes, especially SAPs 5, 6 and 9 mRNA transcripts, in biofilm rather than planktonic cells (Green et al. 2004; Naglik et al. 2008). Recently, in addition to SAPs, a 60 kDa metallopeptidase and 50 kDa serine peptidases have also been isolated, and reported to hydrolyze extracellular matrix proteins and serums (dos Santos et al. 2006). Expression of SAPs has been found to be correlated with other virulence determinants to enhance the pathogenicity of *C. albicans*. Correlation of SAPs with other virulence factors in *C. albicans* is illustrated in Fig. 2.1.

2.6.6 Biofilm Formation

Biofilms are the organized structures involving microbial communities that are attached to some inanimate surfaces or tissues and circumvented in a matrix of

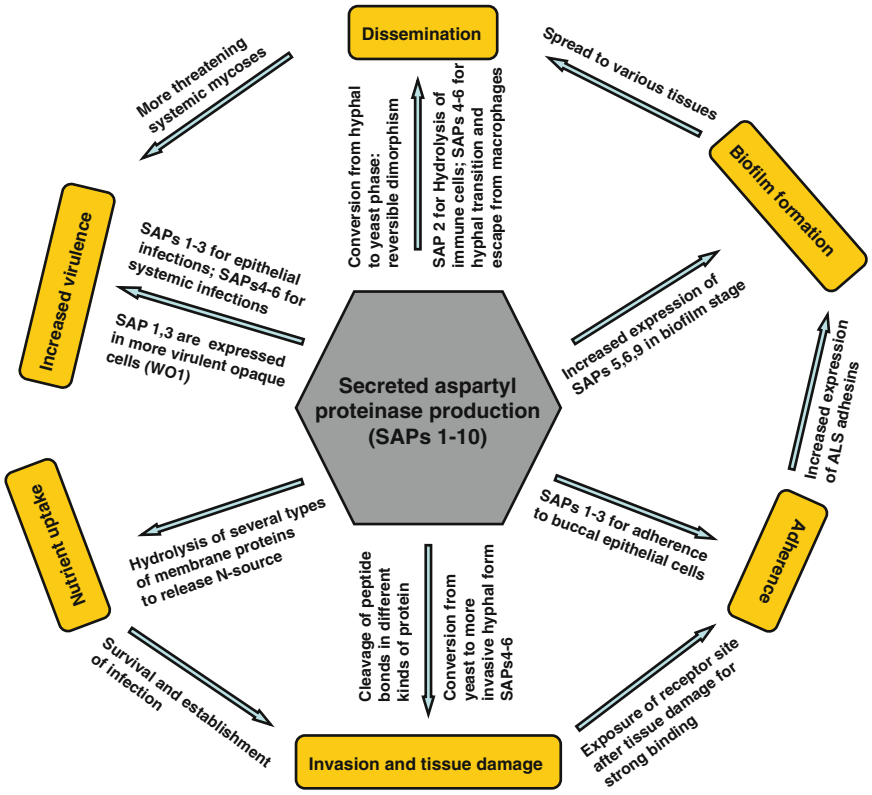

Fig. 2.1 Correlation of secreted aspartyl proteinases with other virulence attributes in *Candida albicans*. Partially adapted from Naglik et al. (2004)

exopolymeric materials. Biofilm formation is initiated by irreversible adherence of microbial cells to tissues or devices and followed by growth and maturation to form a mesh of cells with altered phenotype, growth rate, and gene expression compared to planktonic cells. Studies with scanning electron microscopy of biofilms revealed the presence of both adherent yeast cells and invasive hyphal forms constructing basal and upper layers respectively, enclosed in an extracellular polymer matrix consisting of polysaccharides and proteins and forming a three-dimensional structures with water channels (Dominic et al. 2007). These forms differ in ultrastructure, physiological behavior and composition of cell walls, and are required for candidal pathogenicity, as mutants lacking genes for any one became less virulent both *in vitro* and *in vivo* (Chandra et al. 2001). Heterogeneity of these biofilms depends on the substrate composition, environmental conditions, and type of strains involved. Although yeast–hypha transition is necessary for full maturation of the biofilm, strains that are unable to grow as yeasts or to form hyphae can still form biofilms but are easily detachable (Baille and Douglas 1999). In addition, some authors have reported a change in biofilm-forming ability of candidal cells, with

alteration in variants produced during phenotypic switching (Brown and Gow 1999; Berman and Sudbery 2002).

The ability of *Candida* to form biofilms on catheters, endotracheal tubes, pacemakers and other prosthetic devices has contributed to its predominant prevalence in nosocomial infections (Douglas 2003; Ramage et al. 2005). Such devices, in addition to providing a platform for candidal cells to form biofilm, grow and develop, provide a route through host barrier defenses for dissemination. During weakened immunity, hematogenous dissemination of candidal cells from biofilms to deep-seated organs could occur, resulting in candidemia and septicemia. Recent studies have confirmed biofilm growth in the majority of diseases caused by *Candida* spp (Chandra et al. 2001; Douglas 2003; Chandra et al. 2005). Dental plaque is a well-known example of biofilm formation from *Candida* cells, and is responsible for oral candidiasis. Biofilm formation on such tissues is favored by a high concentration of glucose, serum, and other proteins. Biofilm formation was found to be linked to dimorphism and phenotypic switching, well-known virulence traits for candidal cells (Baille and Douglas 1999; Chandra et al. 2001). Also, alerted phenotypes exhibited reduced susceptibility to the host immune system and to antifungal drug therapy (Chandra et al. 2001). These biofilm-specific cell properties are an indicator for virulence, and have prompted much recent interest in *C. albicans* biofilm structure, physiology, and regulation. Therefore, knowing the ability of *C. albicans* to populate a surface and produce a biofilm as a virulence trait, exhaustive research is being focused on the prevention of biofilm infection by *Candida* cells.

Adherence is the critical property for biofilm-forming cells and is mediated by hydrophobic interactions, electrostatic forces, and adhesion–ligand interactions; multiple adhesion molecules function in the successful establishment of biofilm. A variety of genes are involved in adhesion, and penetrations are associated deeply with the biofilm-forming capability of *C. albicans*. Here, we would discuss some of them for their role in pathogenicity. A number of adhesins, termed glycosylphosphatidylinositol-dependent cell wall proteins (GPI-CWPs), encoded by ALS1, ALS2, ALS4, ALS5 (ALA1), HWP1, and EAP1, mediate adhesion to organic and inorganic surfaces, extracellular matrix proteins, human endothelial cells, and epithelial cells (Blankenship and Mitchell 2006; Filler et al. 2006; Zhao et al. 2006). Experimental results showed upregulated ALS family gene expression in biofilm-forming cells compared with planktonic cells (Hoyer et al. 1998; Chandra et al. 2001; Nobile and Mitchell 2005; Green et al. 2004) and ALS 3 was also found necessary for biofilm formation on silicone substrates *in vitro* (Nobile et al. 2006a). ALS1, ALS3, and HWP1 are regulated by transcription factor BCR1 (biofilm and cell wall regulator), a zinc finger protein, which is under the control of transcription factor Tec1. An als3/als3 mutant strain was found defective in biofilm formation *in vitro*, and overexpression of ALS3 permitted biofilm formation by a bcr1/bcr1 mutant *in vitro* and *in vivo*. Studies with the bcr gene revealed involvement of BCR1 in governing the mechanism of biofilm formation only and not the filamentation (Nobile and Mitchell 2005; Lopez-Ribot 2005; Nobile et al. 2006a; Nobile et al. 2006b). Hwp1 is a cell surface protein covalently linked to the cell wall glucan

through a remnant of its GPI anchor. Functional analysis showed its requirement for tight adherence to oral epithelial cells (Chaffin et al. 1998; Staab et al. 1999; Mendes-Giannini et al. 2008). A role for Hwp1 in *C. albicans* cell–cell adherence is exhibited from the finding that it is induced by mating factor and is deposited on the surface of the bridge between mating partners (Staab et al. 1999; Hoyer et al. 1998). Recent studies showed that Hwp1is required as first cell surface protein *in vivo* for biofilm formation (Nobile et al. 2006a; Nobile et al. 2006b). Recently, work from Granger et al. (2005) described the role of Ywp1 (Yeast cell wall specific protein) as anti-adhesin. The mutant for Ywp1 led to enhanced adherence of yeast cells, therefore highlighting its negative role in biofilm establishment. In addition, studies with mutants for transcription factor Ace2 (activation of CUP1 expression 2) resulted in inhibition of biofilm formation (Kelly et al. 2004). A study conducted by Li et al. (2007) showed eap1 mutants exhibiting reduced adhesion to plastic surfaces and epithelial cells, and that Eap1p was able to mediate adhesion to yeast cells. The same study also showed of the need for eap1 gene expression in biofilm formation under shear flow *in vitro* and in central venous catheter biofilm model *in vivo*. In a study, another cell wall protein Ecm33 was found to be necessary for cell wall integrity and yeast-to-hypha transition (Martinez-Lopez et al. 2004), and Mp65 (Norice et al. 2007) is also required for full virulence in a disseminated infection model, illustrating that cell wall proteins may have diverse functions that are relevant to infection. Norice et al. (2007) also showed that protein SUN41 plays major roles in biofilm formation, cell wall integrity, and virulence in both oropharyngeal and disseminated candidiasis.

Richard et al. (2005) showed involvement of genes sun3, nup85, mds3, kem1 in hyphal development and biofilm formation. Some studies have also demonstrated that the hyphal regulatory gene efg1 is required for normal biofilm growth, and efg1/efg1 and efg1/efg1 cph1/cph1 mutants have exhibited defective biofilms and also adhered poorly to the substrate (Lewis et al. 2002; Watamoto et al. 2009). Gcn4, a general amino acid control regulatory gene, was shown to be required for full biofilm biomass production (Blankenship and Mitchell 2006). A contact-activated protein kinase, Mkc1p, is also required for biofilm development, suggesting that *C. albicans* may respond uniquely to surface contact during biofilm formation.

Further, an experiment by Ramage et al. (2002) showed that biofilms are organized communities under tight regulation of gene expression controlled through quorum sensing which in turn is regulated by farnesol and tyrosol molecules. Several workers have observed these organized communities under the control of a signaling molecule (Hogan 2006). This cell-to-cell communication prevents and controls unnecessary overpopulation and nutritional competition, and has implications in dissemination and establishment of infection at the distal site from old biofilm (Alem et al. 2006). Farnesol, a quorum-sensing molecule that inhibits *C. albicans* biofilm formation by inhibiting yeast-to-hypha transition, decreases HWP1 expression in biofilms (Ramage et al. 2002). Farnesol acts on yeast cells to prevent filamentation but elongated hyphae continue to form biofilms. A report highlighted differential expression of genes associated with hyphae formation in farnesol-treated biofilms, such as genes involved in cell wall maintenance,

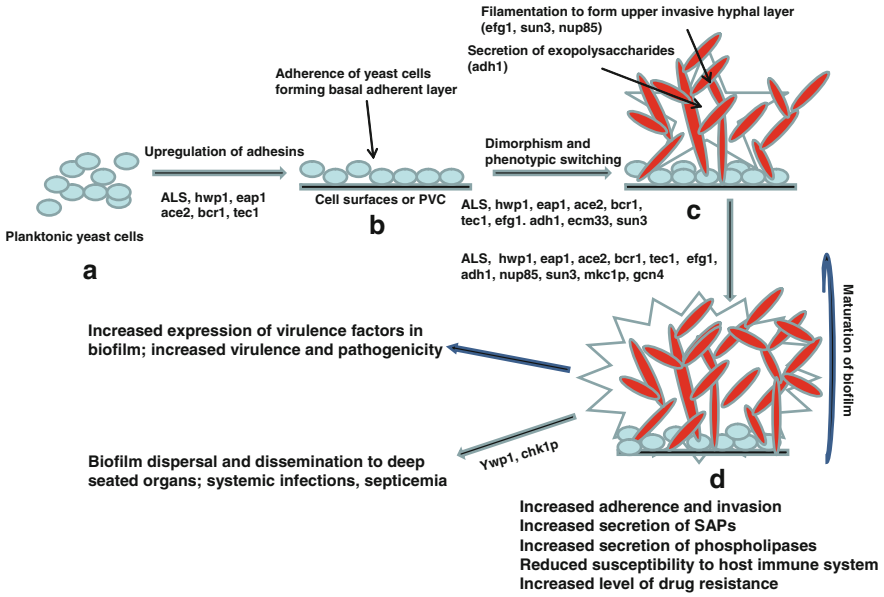

Fig. 2.2 Illustration of virulence factors involved in establishing biofilm and associated pathogenicity in *Candida albicans*: (**a**) planktonic yeast cells, (**b**) initiation of biofilm formation on living tissues or inert object like polyvinyl chloride (PVC) by adherence of yeast cells forming basal layer, (**c**) development of biofilm by initiation of upper invasive hyphal layer and production of exoploysaccharide, and (**d**) maturation of biofilm by more filamentation and exopolysaccharide production. Partially adapted from Blankenship and Mitchell (2006)

iron transport, stress response and upregulation of TUP1 and downregulation of CSH1 protein associated with cell surface hydrophobicity. Farnesol also prevents induction of Tup1-regulated filament specific genes hwp1, rbt1, cph1 and hst7 (Cao et al. 2005; Dominic et al. 2007). In contrast to farnesol, another quorum-sensing molecule, tyrosol, induces filamentation under conditions conducive to germ tube formation, but its role in biofilms has not been much investigated (Alem et al. 2006). However, a recent study highlighted a two-component signal transduction protein Chk1p regulating both quorum sensing and biofilm formation by negatively regulating hyphal development in *C. albicans*. However, it is not clear whether chk1p is directly involved in response to farnesol or not (Kruppa et al. 2004; Blankenship and Mitchell 2006). Involvement of different virulence factors in forming biofilms and associated pathogenicity is depicted in Fig. 2.2.

2.7 Conclusion

Based on the review of the literature on fungal infection, virulence, and pathogenicity, it is clear that at present the frequency of fungal infection rate is increasing, up to 90% for patients with disseminated candidiasis, aspergillosis or

cryptococcosis. Fungal pathogenesis is a multifactorial phenomenon; therefore, the nature of fungal pathogens, their virulence factors, and their interaction with host defense mechanisms need to be explored for the development of more effective antifungal therapy. Although phenomenal progress has been made on molecular characterization of various virulence factors and host–fungi interactions; this issue needs further investigation in order to know the exact contribution of each virulence factor under different disease conditions.

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