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(中、英文) Cloning and characterization of ADP-ribosylation factors in *Candida albicans* and functional analysis of their role in the secretion of aspartyl proteinases (2nd year)

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Cloning and Characterization of ADP-ribosylation Factors from *Candida albicans* and Functional Analysis of their Roles in the Secretion of Aspartyl Proteinases (second year)

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中文摘要

白色念珠菌(*Candida albicans*) 已成爲人類重要致病菌，會帶給病人高死亡率及併發症。然國內之研究相當缺乏，尤其是致病機轉之研究。本計畫乃針對 ADP-ribosylation factor (簡稱 ARF) 可能在蛋白質傳輸，包括致病因子 aspartyl proteinases 分泌之調控，所扮演的功能進行研究。白色念珠菌 ARF 基因已被選殖，並利用 Ura-blaster 方法進行系列破壞白色念珠菌 SC5314 菌株雙套染色體之 2 個 alleles 之 ARF 基因。此外，本研究以間接螢光染色法定出 Arf 蛋白在白色念珠菌細胞內之分布以推測其可能之功能。利用 anti-yArf1 多株抗體染色，白色念珠菌 yeast cells 的細胞質上有點狀螢光，像酵母菌細胞內之分布。然而在念珠菌有菌絲形成時，可在母細胞及菌絲之間看見較強的染色。其分布與先前之推測相吻合，也就是 Arf 蛋白可能在蛋白質傳輸扮演某些功能。

Summary

Candida spp. have assumed increasing prominence as major human pathogens which cause significant morbidity and mortality. The incidence of nosocomial *Candida*l infections, particularly nosocomial candidemia, increased dramatically in the past decade and in the United States as well as in Taiwan. However, little, if any, studies exploring pathogenesis of candidiasis is conducted in Taiwan. Several potential virulence factors in *Candida* have been examined. Among them, **secreted aspartyl proteinases** from ***C. albicans***, the most important pathogen of candidiasis, raise more intention recently. Knowledge of virulence factors or mechanisms of regulation or secretion of these virulence factors can be applied to develop strategies for new antifungal agents. Recently, the secretion pathway has provided a valid target for antibacterial chemotherapy. Considering the molecules involving protein secretion, **ADP-ribosylation factors** (**Arfs**) are highly conserved ~20 kDa guanine nucleotide-binding proteins that enhance the ADP-ribosyltransferase activity of cholera toxin, and are believed to participate in vesicular transport in both endocytic and exocytic pathways.

During the prior project period, immunoblotting analysis identified three *Candida* proteins, which are immunologically related to *S. cerevisiae* Arf proteins (yArf1, yArf2 and yArl1). Besides, the expression levels of assumed Arf2, Arl1 and Arf1 proteins were the same between the majority of *Candida* species tested. They included four leading pathogens of candidemia (*C. albicans, C. parapsilosis, C. tropicalis, C. glabrata*), as well as three emerging pathogens (*C. guillemondii,*

C. krusei, C. lusitaniae). This data confirm that Arfs are highly conserved throughout evolution.

During the current project year, *ARF* gene of *C. albicans* was cloned. In order to define the role of Arf proteins in the secretion of aspartyl proteinases, arf null mutants were constructed by Ura-blaster technique for sequential disruption of two alleles of *ARF* gene of *C. albicans strain* SC5314 by integrative transformation using *URA3* as a selectable marker. As the cellular localization of cArf1 could provide hints regarding its function, indirect immunofluorescence staining of *C. albicans* was done. Using anti-yArf1 polyclonal antibody, a punctate staining in the cytoplasms of *C. albicans* yeast cells was observed. The picture was similar to that obtained in *S. cerevisiae*. There was no perinucleaar staining typical for ER localization. On the other hand, *C. albicans* with hypha formation showed intense staining near the junction of mother cell and hyphae. It is compatible with the hypothesis that Arf protein might participate in protein transport.

Key words: *Candida albicans*, ARF, ADP-ribosylation factor, secreted aspartyl proteinase, virulence factor, protein transport

Introduction

Patients with candidiasis increasing worldwide. *Candida* spp. have assumed increasing prominence as major pathogens in medical centers as well as in community hospitals. The incidence of nosocomial *Candida*l infections increased dramatically in the past decade in the United States as well as in Taiwan. They cause excessive length of hospitalization and carry a significantly high case fatality rate, particularly in nosocomial candidemia (up to 50%) (1-3). *Candida* became the leading blood culture isolates in several medical centers in the Untied States (4) and was the most common pathogens causing nosocomial bloodstream and urinary tract infections at National Taiwan University Hospital since 1993 (5). However, little, if any, studies exploring pathogenesis of candidiasis is conducted in Taiwan.

A secreted virulence factor in *Candida*. A growing list of potential virulence factor in *Candida* has been examined (6). Among them, secreted aspartyl proteinases (Saps) in *C. albicans* raises more intention recently (7,8). Comparing different *Candida* species, aspartyl proteinases are secreted almost exclusively by pathogenic strains of *Candida* either in culture or at the infectious sites, but not by nonpathogenic *Candida* species, and its potential role in the host-pathogen interactions has been explored (10-12). Recently, disruption mutants of several *SAP* gene family have been constructed and demonstrated attenuated virulence in animal models (33,34). As we know, despite of antifungal treatment and aggressive intensive care, the attributable mortality and morbidity

of candidemia remained high. Development of new antifungal agents with less toxicity and potent fungicidal effects, and, if possible, against new target to overcome the emergence of resistance to present antifungal agents is warranted. Knowledge of virulence factors or mechanisms of regulation or secretion of these virulence factors can be applied to develop strategies for new antifungal agents. As renin and the protease of HIV, other members of aspartyl proteinases family, have been intensely studied and proved to be a useful target for drug design (9), In addition, the secretion pathway has provided a valid target for antibacterial chemotherapy recently (28-30). Saps proteins and their secretion pathway, provide a **new target of antifungal agents**.

ADP-ribosylation factors (Arfs). Considering the molecules involving protein secretion, ADP-ribosylation factors (Arfs) are believed to participate in vesicular transport in both endocytic and exocytic pathways (13-15). The Arf family comprises a group of structurally and functionally conserved ~20 kDa guanine nucleotide-binding proteins in eukaryotes, which are members of the Ras superfamily of regulatory GTP-binding proteins (13). Arfs have been found to have a number of disparate activities, including the regulation of membrane traffic in both endocytic and exocytic pathways and play an important role in vesicle transport (13-15). The Arf family is divided functionally into the Arf and Arf-like (Arl) proteins. Arfs can be distinguished from Arls by their ability to act as cofactors for cholera toxin-dependent ADP-riboxylation of the heterotrimeric G protein G_s, and ability to rescue lethal, double *arf1-arf2-* deletion in the *Saccharomyces cerevisiae* (13).

Arfs appear to cycle between an active, membrane-bound state when GTP or the non-hydrolyzable analogue guanosine 5' (γ-thio)triphosphate (GTPγS), is bound, and an inactive, soluble state when bound GTP is hydrolyzed to GDP, with assistance of a GTPase-activating protein (GAP). Conversion of Arf-GDP to Arf-GTP is promoted by a guanine nucleotide-exchange protein (GEP) (16). Brefeldin A (BFA), a fungal metabolite, reversibly causes rapid disintegration of Arf from the Golgi membranes by affecting GDP/GTP exchange in cells (17). Previous study identified a Arf protein (PfArf1) predominantly expressed in the ring stage of *Plasmodium falciparum* (18) and some evidence suggest that BFA also affects the transport of most parasitic proteins in the erythrocyte (19).

Using polymerase chain reaction-based techniques and molecular cloning from cDNA and genomic libraries, a **multigene family** was identified that includes three *ARF* genes in *S. cerevisiae* and six in mammalians (13,20). Based on size, phylogenetic analysis, amino acid sequence, and gene structure, the mammalian Arfs fall into three classes: class I (Arfs 1,2, and 3) with 181 amino acids, class II (Arfs 4 and 5) with 180 amino acids, and class III (Arf6) with 175 amino acids. They share 65-96% amino acid identity and contain consensus sequences believed to involve in quanine nucleotide binding and GTP hydrolysis (phosphate binding) (20). In addition, they have glycine at position 2 (the site of *N*-myristoylation), lack the Cys-Ala-Ala-Xaa box motif (site of C-terminal carboxypeptidase cleavage, carboxymethylation, and prenylation in most members of the Ras superfamily).

Yeast Arf proteins, along with *ypt1*- and *rab*-related gene products, have been implicated in vesicular transport, including budding from the endoplasmic reticulum and fusion at the Golgi, endosome, and nuclear membranes (13-15). It seemed likely that the several mammalian Arfs should have different functions and intracellular localizations (21). Consistent with this view, Arf1, 3, and 5 proteins clearly differed in their binding to a Golgi fractions well as in their dependence on accessory proteins for interaction with Golgi and, perhaps, other cellular membranes.

Targeted mutagenesis. Many *C. albicans* genes were first cloned by complementation of mutations in *S. cerevisiae* counterparts or conferring new attributes to the latter. Regarding the evolutionary distance between *S. cerevisiae* and *C. albicans*, and the fact that *C. albicans* is an extremely common pathogen, and *S. cerevisiae* is only rarely associated with clinical disease, it is likely that expression of these genes in vivo might be varied and some of the homologous genes might do different things in different host (31). For example, the pathway in *S. cerevisiae* that is used for mating reaction is used in *Candida* to signal transduction to a filamentous form. Therefore, exploring these genes will point to special adaptation for the commensal and ultimately the pathogenic life style. Direct mutagenesis of target gene is required to explore the role of *ARF*1 gene in protein transport. The fact that the organism is diploid and has no known sexual cycle has hindered the genetic study of potential virulence factors of this increasingly common cause of serious infections. Despite these practical difficulties, the recent years have seen a number of advances in the molecular

genetics of *C. albicans*, including methods for transformation, the construction of autonomously replicating plasmids, and a technique for gene disruption involving convenient inactivation of both alleles of a gene (31).

Objectives. In *C. albicans*, two *ARF* genes are isolated and sequenced (GenBank accession No. M54910 and No. P22274) (13,22). However, little is known about their cellular function. Our goal in this project was to construct ARF disruption mutants *C. albicans* and to define the role of Arf proteins in the transport of secreted aspartyl proteinases in *C. albicans*.

Materials and Methods

Strains, media, and culture conditions. The *C. albicans* strains used in this study were SC5314, CAI4 and CAI12. CAI4 and CAI12, obtained from Dr. WA Fonzi (25), are homozygous *ura3* null mutant and *URA3* revertant strain, respectively, and are isogenic to SC5314, a clinical isolate from a patient with disseminated candidiasis. As more and more researchers using CAI4 to construct disruption mutants of their interested genes, the genetic background of SC5314 is elucidated gradually. All strains were routinely propagated in YEPD (2% Bacto-Peptone [Difco Laboratories, Detroit, Mich.], 1% yeast extract, 2% glucose), or synthetic dextrose (SD) (0.7% yeast nitrogen base without amino acids, 2% glucose) agar or medium at 30°C. Agar was added (2%) for solid media. Yeast cells were induced to form germ tubes in fetal bovine serum or RPMI 1640 with L-glutamin at 37°C for 2 hr. Media were supplemented with 50 ug of uridine per ml as required. Selection of auxotrophs was on SD medium containing 5-fluoroorotic acid (5-FOA).

pET15b vector (Novagen, Inc., WI, USA) was used for cloning and expression of fusion protein in *E. coli*. *E. coli* DH5α(*recA*-) was used as a host for plasmid construction and *E. coli* BL21 (F^-*ompT hsd* S_B ($r_B^-m_B^-$)*gal dcm* (DE3)) as a host for expression of fusion protein for rapid affinity purification. *Candida* and *E. coli* were cultivated aerobically at 30°C and 37°C on a rotary shaker (200 rpm), respectively.

PCR cloning of *ARF* gene family other than *ARF1*. The protocol used for PCR amplification was described previously (20). Primers were designed based on concensus sequences of *yARF* gene family. PCR with a specific primer, similar to rapid amplification of cDNA ends-PCR (RACE-PCR)(23), was used to isolate the 3' and 5' ends of the *ARF* analogue. After the complete *cARF* cDNA was obtained, DNA sequences was analyzed by deoxy chain-termination method (24).

Chromosomal location of *cARF1*. Chromosomal DNA was resolved by pulsed-field gel electrophoresis with a contour-clamped homogeneous electric field system (BioRad) and transferred to a nylon membrane. The probe and hybridization methods were the same as described in Southern blot analysis.

Northern blot quantification of *Candida* total RNA. Total RNA from mid-log *Candida* cultures grown in YPD medium was isolated by the guanidinium thiocyanate/phenol-chloroform method, separated on an agarose gel containing formaldehyde, and transferred and hybridized as described above. Before transfer, gels were stained with ethidium bromide in order to ascertain that equal amounts of total RNA have been separated. For quantitative analysis of the signals, the membranes will be exposed for several hours to a phosphor screen, which is subsequently analyzed by a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

Plasmid construction and expression of recombinant cArf proteins in *E. coli*. For the 6-His-tagged cArf fusion proteins, DNA fragments containing the coding regions of *cARF*1 (GenBank accession No. M54910; open reading frame,

395-934 nt) and newly identified *cARFs* was generated by amplifying *Candida* cDNA library with the sequence-specific primers that incorporate restriction sites for transfer to plasmid pET15b (Novagen; Madison WI, USA). *E. coli* BL21 (DE3) competent cells was transformed with recombinant plasmid, and grown on LB plates containing ampicillin (100 ug/ml). To identify colonies that have the highest expression of the target protein, transformants (2 ml each) were grown in 6-well plates for 3 hr before induction with isoprophyl-D-thio-galactopyranoside (IPTG) (final concentration, 0.5 mM) for 2 h. The total lysate from a 100-μl sample of each transformant was first assessed by analysis on sodium dodecyl sulfate (SDS) polyacrylamide gel (PAGE) under reducing condition, followed by Coomassie blue staining. For large-scale expression, 25 ml of overnight culture was used to inoculate 500 ml of LB broth containing ampicillin (100 ug/ml), and protein expression was be induced with 0.5 mM IPTG for 3 h after OD600 reached around 0.5. The distribution of target protein in soluble fraction and insoluble fraction was determined before affinity purification.

Rapid affinity purificaiton of cArf fusion proteins. pET15b carries the His • Tag sequence which binds to Ni^{2+} immobilized on the His • Bind metal chelatin resin, after unbound proteins are washed away, the target protein was recovered by elution with imidazole gradient. Target protein was purified from soluble fraction in native form and from insoluble fraction under denaturing condition by adding urea (final concentration 6M) to binding buffer, wash buffer and elute buffer. Protein concentration was determined by Coomassie Blue dye-

binding assay (Bio-Rad), and purity was assessed by SDS-PAGE followed by Coomassie Blue staining.

Antisera production and immunoblotting. Polyclonal antisera against cArf1, yArf1, yArf2, yArl and yArp were raised by immunizing rabbits with the fusion proteins expressed in *E. coli*. Protein samples prepared from clinical isolates of *C. albicans* and other *Candida* species was separated by 12% SDS-PAGE, transferred onto a nylon membrane, and incubated with the antisera at a dilution fo 1/1,000. Immunoreactivity was detected using the ECL kit (Amersham's Corp.) according to manufacturer's instructions. For blocking of the Arf-specific antisera, 100μg of serum was incubated with certain amount of recombinant fusion proteins at 4°C overnight.

Subcellular localization. To determine the intracellular localization of cArf protein, we construct an HA-epitope-tagged version of cArf fusion protein. Western analysis using anti-HA antibody should show that the recombinant protein migrated as a 20 kDa band, which could not be detected in strains containing only the untagged cArfp. Indirect immunoflourescence using the anti-HA monoclonal antibodies and polyclonal antibodies against cArfp was used.

***C. albicans ARF1* gene cloning and disruption**. Gene disruption mutants were constructed by Ura-blaster technique for sequential disruption of multiple alleles by integrative transformation using *URA3* as a selectable marker (25). Full-length *C. albicans ARF1* gene was amplified by PCR using SC5314 genomic DNA as template. Primers were designed based on *C. albicans ARF1* gene (GeneBank

accession number M54910) to amplify the 540-bp coding region and introduce *Nde*I and *BamH*I sites at either ends. PCR product was cloned into *NdeI/BamH*I sites of pET15b. This plasmid was then digested with *Alu*I to delete 216-bp fragment and replaced by a blunt-ended *KpnI/HindIII* fragment of pMB-7, which contains the *C. albicans URA3* gene flanked by direct repeats of *Salmonella hisG* DNA (25). The resulting plasmid, designated pYC11, was digested with *Nde*I and *BamH*I to release the insert (ARFD cassette), which was used to transform CAI4 by a lithium acetate method (32). Selection of Ura3$^-$ isolates resulting form *cis* recombination between the *hisG* repeats was facilitated by plating transformants onto synthetic dextrose agar plate containing 0.1% (w/v) 5-FOA and 50 ug of uridine per ml. The disruption transformants were repeated until a null mutants was generated. All isolated were verified by Southern blot analyses.

Southern blot analysis of *Candida* genomic DNA. *Candida* genomic DNA was isolated (26), and either directly subjected to pulse-field gel electorphoresis on a CHEF-DRIII unit (Bio-RAD; Hercules, CA. USA), or after digestion with restriction endonuclease *Xba*I or *Sfi*I, subjected to agarose gel electrophoresis. DNA was transferred to a nylon membrane, hybridized with a random-primed ^{32}P-labeled probe for 16h, washed. and autoradiographed (27). The *cARF* probes was a 540-bp DNA fragment amplified by PCR from genomic DNA. Blotting was carried out with Hybond-N nylon membranes (Amersham, Chicago, Ill.) as described in the manufacturer's instructions.

Determination of growth rate of arf mutants. *C. albicans* strains were grown in YEPD medium with or without supplemented uridine at 30°C on a rotaray shaker (200rpm) for 24h. The cells were then washed in PBS and counted with a hemacytometer. The cell suspensions were adjusted to 10^4cells/ml and used to inoculate 100 ml YEPD to a final optical density of 0.01-0.02 at 600nm. The OD_{600} was measured every hour until the stationary phase of the growth curve was reached. The doubling time during the log phase was determined by the formula: ln(2xt)/(lnb-lna) (t = time period in hour; a = OD_{600} at the beginning of time period; b = OD_{600} at the end of time period) (37).

Morphogenesis of C. albicans *arf* mutants. The cells were incubated on albumin-containing media for over 10 days and the edge of the colonies was observed. Microscopic examination was done to verify the morphology of the cells. Induction of germ tube formation was accomplished by growing yeast in YEPD at 30°C for 24h (stationary-phase), washed in water, and then transferring to the defined tissue culture medium RPMI 1640 with L-glutamine (RPMI; Gibco BRL catalogue number 11875), YEPD with 5% fetal bovine serum (Life Technologies, Inc.), or Medium 199 (Gibco) to achieve approximately 10^6 cells/ml and incubating at 37°C with shaking (200rpm) for 24h. The proportion of germ tube formation and the length of germ tube were measured at 2h, 4h, 6h, and 24h. Moreover, the strains grew under hyphae-inducing conditions was stained with calcofluor white to demonstrate the deposition of chitin, which normally most intensely focused on sites of budding, in bud scars, and in septa between cells (35).

Effects of *arf* mutants on proteinase secretion. *C. albicans* proteinase secretion was induced as described (36); organisms were grown at 25°C to prevent the formation of hyphae. Plate assays for proteinase production incorporated 1% agarose in the standard media and were enhanced by staining with 0.1% Amido Black in acetic acid/methanol/ H2O (10:25:65), followed by destaining in acetic acid/methanol/water. Proteinase secretion in yeast phase and in hyphae-induction media (see above) was evaluated at 2h, 4h, 6h and 24h after induction.

Results

Construction of the *C. albicans ARF1* null mutant. Plasmids for gene disruption were constructed. Now the preparation of *C. albicans arf1* mutants is still in progress.

Indirect immunofluorescence to localize cArf1. As the cellular localization of cArf1 could provide hints regarding its function, indirect immunofluorescence staining of *C. albicans* was done. Several growth conditions were evaluated. *C. albicans* SC5314 was fixed with formaldehyde. Spheroplasts were prepared by zymolase digestion of cell walls. Using anti-yArf1 polyclonal antibody, a punctate staining in the cytoplasms of *C. albicans* yeast cells was observed. The picture was similar to that obtained in *S. cerevisiae*. There was no perinucleaar staining typical for ER localization. On the other hand, *C. albicans* with hypha formation showed intense staining near the junction of mother cell and hyphae. It is compatible with the hypothesis that Arf protein might participate in protein transport.

Discussion

Our study showed that *Candida* Arf proteins are immunologically related to 3 *S. cerevisiae* Arf proteins. Besides, the expression levels of Arf2, Arl1 and Arf1 were the same between different *Candida* species. expression of this putative *Candida* ARF1 gene apparently was different when we tested several medically important *Candida* clinical isolates, including *C. albicans, C. glabrata, C. tropicalis* and *C. parapsilosis.* This data supported that Arfs are highly conserved throughout evolution (13).

Considering the evolutionary distance between *S. cerevisiae* and *C. albicans*, and the fact that *C. albicans* is an extremely common pathogen and *S. cerevisiae* is only rarely associated with clinical disease, it is likely that expression of these genes might be varied. In addition, some of the homologous genes might do different things in different host. For example, the pathway in *S. cerevisiae* that is used for mating reaction is used in *Candida* to signal transduction to a filamentous form. Therefore, exploring these genes will point to special adaptation for the commensal and ultimately the pathogenic life style. Our result demonstrated that there was higher expression of Arl1 in *C. parapsilosis,* and Arf2 in *C. glabrata* and *C. guillermondii.* There raised a possibility that Arf proteins might be of clinical relevance and can play a role in the regulation of protein secretion. Besides, the secretion pathway has provided a valid target for antibacterial chemotherapy recently (28-30). Direct mutagenesis of *ARF1* gene is now undergoing to explore the role of *ARF*1 gene in the protein secretion

pathway. Null mutants were constructed by Ura-blaster technique for sequential disruption of two alleles of *ARF* gene of *C. albicans strain* SC5314 by integrative transformation using *URA3* as a selectable marker. As the cellular localization of cArf1 could provide hints regarding its function, indirect immunofluorescence staining of *C. albicans* was done. Using anti-yArf1 polyclonal antibody, a punctate staining in the cytoplasms of *C. albicans* yeast cells was observed. The picture was similar to that obtained in *S. cerevisiae*. There was no perinucleaar staining typical for ER localization. On the other hand, *C. albicans* with hypha formation showed intense staining near the junction of mother cell and hyphae. It is compatible with the hypothesis that Arf protein might participate in protein transport.

References

1. Beck-Saque CM, Jarvis WR, the National Nosocomial Infections Surveillance System. Secular trends in the epidemiology of nosocomial fungal infections in the United States, 1980-1990. J Infect Dis 1993;167:1247-51.
2. Hung CC, Chen YC, Chang SC, Luh KT, Hsieh WC. Nosocomial candidemia in a university hosptial in Taiwan. J Formos Med Assoc 1996;95:19-28.
3. Wey SB, Mori M, Pfaller MA, Woolson RF, Wenzel RP. Hospital-acquired candidemia - the attributable mortality and excess length of stay. Arch Intern Med 1988;148:2642-5.
4. Wilson ML, Davis TE, Mirrett S. et al. Controlled comparison of the BACTEC high-blood-volume fungal medium, BACTEC plus 26 aerobic blood culture bottle, and 10-milliliter isolator blood culture system for detection of fungemia and bacteremia. J Clin Microbiol 1993;31:865-71.
5. Chen YC, Chang SC, Sun CC, Yang LS, Hsieh WC, Luh KT. Secular trends in the epidemiology of nosocomial fungal infection at a teaching hospital in Taiwan, 1981-1993. Infect Control Hosp Epidemiol 1997 May (in press)
6. Perfect JR. Fungal virulence genes as targets for antifungal chemotherapy. Antimicrob Agent Chemother 1996;40:1577-83.
7. Agabian N, Odds FC, Poulain D, Soll DR, White TC. Pathogenesis of invasive candidiasis. J Med Veter Mycol 1994;32(Suppl 1):229-37.
8. White TC, Agabian N. *Candida albicans* secreted aspartyl proteinases: isoenzyme pattern is determined by cell type, and levels are determined by environmental factors. J Bacteriol 1995;177:5215-21.

9. Balter M. New hope in HIV disease. Science 1996;274:1988.

10. Colina AR, Aumont F, Deslauriers N, Belhumeur P, de Repentigny L. Evidence of degradation of gastrointestinal mucin by *Candida albicans* secretory aspartyl proteinase. Infect Immunol 1996;64:4514-9.

11. Kaminishi H, Miyaguchi H, Tamaki T, Suenaga N, Hisamatsu M, Mihashi I, Matsumoto H, Maeda H, Hagihara Y. Degradation of humoral host defense by *Candida albicans* proteinase. Infect Immunol 1995;63:984-8.

12. Tsushima H, Mine H. Cleavage of human big endothelin-1 by *Candida albicans* aspartic proteinase. FEMS Immmun Med Microbiol 1995;11:69-72.

13. Boman AL, Kahn RA. Arf proteins: the membrane traffic police? TIBS 1995;20:147-50.

14. Rothman JE, Wieland FT. Protein sorting by transport vesicles. Science 1996;272:227-34.

15. Moss J, Vaughan M. Structure and function of ARF proteins: activators of cholera toxin and critical components of intracellular vesicular transport processes. J Biol Chem 1995;270:12327-30.

16. Morinaga N, Tsai SC, Moss J, Vaughan M. Isolation of a brefeldin A-inhibited guanine nucleotide-exchange protein for ADP ribosylation factor (Arf)1 and Arf3 that contains a *Sec7*-like domain. Proc Natl Acad Sci USA 1996;93:12856-60.

17. Donaldson JG, Finazzi D, Klausner RD. Brefeldin A inhibits Golgi membrane-catalysed exchange of guanine nucleotide onto ARF protein. Nature 1992;360:350-2.

18. Lee FJS, Patton WA, Lin CY, Moss J, Vaughan M, Goldman ND, Syin C. Stage-specific expression of an ADP-ribosylation factor gene in *Plasmodium falciparum*. (submitted)

19. Benting J, Mattei D, Lingelbach K. Brefeldin A inhibits transport of the transport of the glycophorin-binding protein from *Plasmodium falciparum* into the host erythrocyte. Biochem J 1994;300:821-6.

20. Lee FJS, Stevens LA, Kao YL, Moss J, Vaughan M. Characterization of a glucose-repressible ADP-ribosylation factor 3 (ARF3) from *Saccharomyces cerevisiae*. J Biol Chem 1994;269:20931-7.

21. Cavenagh MM, Whiney A, Carroll K, Zhan CJ, Boman AL, Rosenwald G, Mellman I, Kahn RA. Intracellular distribution of Arf proteins in mammalian cells. J Biol Chem 1996;271:21767-74.

22. Denicyh KT, Malloy PJ, Feldman D. Gene 1992;110:123-8.

23. Frohman MA, Dush MK, Martin GR. Proc Natl Acad Sci USA 1988;85:8998-9002.

24. Sanger F, Nicklen S, Coulson RA. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 1977;74:5463-7.

25. Fonzi WA, Irwin MY. Isogenic strain construction and gene mapping in *Candida albicans*. Genetics 1993;134:717-28.

26. Lee FJS. Modified protocol for yeast DNA mini-preparation. BioTechniques 1992;12:377

27. Lee FJS, Moss J, Lin LW. BioTechniques 1992;13:844-6.

28. Alksne LE, Burgio P, Bradford P, et al. Identification of inhibitors of bacterial secretion by using a SecA reporter system. 38th ICAAC Abstract F-157; 1998

29. Alksne LE, Burgio P, Barbieri L, et al. Analysis of a series of potential bacterial protein secretion inhibitors. 38th ICAAC Abstract F-158; 1998

30. Yuan H, Gallant P, Shen X, Tao J. Bacterial SecA as an antimicrobial target. 38th ICAAC, Abstract F-162;1998

31. Magee PR, Scherer S. Genome mapping and gene discovery in *Candida albicans*. ASM News 1998;64:505-11.

32. Gietz D, Schiestl RH, William AR, Woods RA. Studies on the transformation of intact yeast cells by the LiAc/ss-DNA/PET procedures. Yeast 1995;11:355-60.

33. Hube B, Sanglanrd D, Odds, FC, et al. Disruption of each of the secreted aspartyl proteinase genes *SAP1*, *SAP2*, and *SAP3* of *Candida albicans* attenuates virulence. Infect Immun 1997;65:3529-38.

34. Sanglard D, Hube B, Monod M, Odds FC, Gow NAR. A triple deletion of the secreted aspartyl proteinase genes *SAP4*, *SAP5*, and *SAP6 of Candida albicans* causes attenuated virulence. Infect Immun 1997;65:3539-46.

35. Newport G, Agabian N. Kex2 influences *Candida albicans* proteinase secretion and hyphal formation. J Bio Chem 1997;272:28954-61.

36. Crandall M, Edwards JE Jr. J Gen Microbiol 1987;133:2817-24.

37. Willett HP. Physiology of Bacterial Growth. Section One, Chapter 5 in Zinsser's Microbiology. Joklik, Willett, Amos. 17th Edition, Appleton-Century-Crofts., N.Y.

38. Lay J, Henry K, Clifford J, Koltin Y, Bulawa CE, Becker JE. Altered expression of selectable marker *URA3* in gene-disrupted *Candida albicans* strains complicated interpretation of virulence studies. Infect Immun 1998;66:5301-6.