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CLONAL TYPES OF ORAL YEASTS IN RELATION TO AGE, HEALTH, AND GEOGRAPHY

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Academic Dissertation

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CONTENTS

ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
APECED	autoimmune polyendocrinopathy-candidosis-ectodermal dystrophy
AP-PCR	arbitrarily primed polymerase chain reaction
CCUG	Culture Collection, University of Göteborg, Sweden
CFU	colony-forming unit
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic acid
HIV	human immunodeficiency virus
NCPF	National Collection of Pathogenic Fungi
PCR	polymerase chain reaction
RAPD	randomly amplified polymorphic DNA
REA	restriction endonuclease analysis
REP-PCR	repetitive extragenic palindromic-polymerase chain reaction
RFLP	restriction fragment length polymorphism
SDA	Sabouraud dextrose agar
TSBV	tryptic soy serum bacitracin vancomycin

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which will be referred to in the text by their Roman numerals:

I Hannula J, Saarela M, Jousimies-Somer H, Takala A, Syrjänen R, Könönen E, Asikainen S. Age-related acquisition of oral and nasopharyngeal yeast species and stability of colonization in young children. Oral Mirobiol Immunol 1999: 14: 176-182.

II Hannula J, Saarela M, Alaluusua S, Slots J, Asikainen S. Phenotypic and genotypic characterization of oral yeasts from Finland and the United States. Oral Microbiol Immunol 1997: 12: 358-365.

III Hannula J, Dogan B, Slots J, Ökte E, Asikainen S. Subgingival strains of *Candida albicans* in relation to geographical origin and occurrence of periodontal pathogenic bacteria. Oral Microbiol Immunol, (submitted).

IV Hannula J, Saarela M, Dogan B, Paatsama J, Koukila-Kähkölä P, Pirinen S, Alakomi H-L, Perheentupa J, Asikainen S. Comparison of virulence factors of oral *Candida dubliniensis* and *Candida albicans* isolates in healthy persons and patients with chronic candidosis. Oral Microbiol Immunol, (in press).

INTRODUCTION

Yeasts are opportunistic pathogens and common members of the normal oral flora in humans. However, the source of early yeast infection and time and stability of colonization of yeast species are poorly understood. Yeasts have attracted growing interest among researchers due to the increased incidence of severe oral candidosis. The condition mainly results from the widespread use of antibiotics and rising proportions of aged and immunocompromised individuals in the population. *Candida albicans* is the most common yeast species in the human oral cavity. Immunocompromised individuals are, however, prone to infections by otherwise rare yeast species, such as *Candida dubliniensis, Candida tropicalis, Candida parapsilosis, Candida krusei, Candida kefyr, Candida glabrata,* and *Candida guilliermondii*. Among these, *C. dubliniensis* is a new, recently described species which was originally described from severe oral candidosis of subjects infected with human immunodeficiency virus (HIV) and was therefore suspected of increased virulence.

In the oral cavity, yeasts can be found on mucosal surfaces and in saliva but also occasionally in inflamed periodontal pockets. However, their role in periodontal destruction is unknown. Subgingival yeasts may be merely innocent bystanders and only reflect colonization on oral surfaces. In some individuals the subgingival ecosystem may favor yeasts or enhance growth of certain yeast strains since yeast species or strains may vary in their ability to coaggregate with subgingival bacteria and to survive in the low oxyxen tension of the periodontal pocket. Thus, different yeast species or strains may colonize different areas in the oral cavity. Today, limited data exist on the interindividual and intraindividual heterogeneity of oral yeast species or strains, especially in systemically healthy subjects. Previous studies mainly included either immunocompromised individuals or only a single yeast isolate within a subject. Analyses of several strains within subjects are important, since even among strains of the same species virulence characteristics may vary.

In the present study, phenotypic and genotypic techniques were used to determine clonal diversity of oral yeast isolates from children and adults. The timing, stability, and infection route of oral yeast colonization in healthy young children up to the age of two years were addressed. To identify strains with increased pathogenic potential, clonal distribution and heterogeneity of oral *C. albicans* species was first determined between isolates recovered from geographically distant locales and systemically healthy subjects and then compared to those of isolates from subjects especially susceptible to yeast infections. Serotypes and genotypes of *C. albicans* isolates from periodontal pockets were related to subgingival ecology as assessed by the co-existence of various periodontopathogenic bacteria. Finally, certain virulence attributes were compared within *C. albicans* species and between *C. albicans* and *C. dubliniensis* species.

REVIEW OF THE LITERATURE

THE ORAL CAVITY AS A HABITAT OF YEASTS

The oral cavity comprises diverse surfaces and microenvironments enabling colonization of a wide variety of microorganisms. Mucous membranes line the oral surfaces, which are pierced by teeth and ducts of the salivary glands. The flushing action of saliva removes non-adherent microorganisms from oral surfaces. Decreased saliva secretion (Parvinen & Larmas 1981, Meurman & Rantonen 1994, Almståhl et al. 1999) may result in increased recovery of yeasts, perhaps due to decreased quantities of candidacidal molecules, including lysozyme (Tobgi et al. 1988), histatins (Jainkittivong et al. 1998), peroxidases (Lenander-Lumikari 1992), and lactoferrin (Nikawa et al. 1993). On the other hand, saliva also contains microbial nutrients, such as glucose (Knight & Fletcher 1971), which facilitate proliferation of yeasts (Samaranayake et al. 1986a), and mucins, which contribute to adhesion of yeasts to oral surfaces (Hoffman & Haidaris 1993).

Candida species grow best under aerobic conditions but are also able to grow under elevated concentrations of CO_2 in air (Webster & Odds 1986). Reports on the ability of *Candida albicans* to grow in anaerobiosis have been equivocal (Kennedy 1981, Samaranayake et al. 1983, Webster & Odds 1986). Some oral sites with low oxygen tension, such as buccal folds and periodontal pockets (Eskow & Loesche 1971, Loesche et al. 1983), may not be favorable for the proliferation of *Candida*. Anaerobes grow at low oxygen tension (Loesche et al. 1983), negative oxidation-reduction potential (Kenney & Ash 1969), and basic pH (Eggert et al. 1991), an environment unfavorable for growth of yeasts.

C. albicans can form on oral surfaces an adherent biofilm together with other oral microbiota and host glycoproteins (Holmes et al. 1995). Coaggregation of *Candida* with oral bacteria is believed to play an important role in the establishment of candidal colonization and candidosis (Bagg & Silverwood 1986). *C. albicans* binds to bacteria such as viridans streptococci (Jenkinson et al. 1990, Holmes et al. 1996), *Fusobacterium* (Grimaudo & Nesbitt 1997), and *Actinomyces* species (Grimaudo et al. 1996). Coaggregation of yeast and streptococci may be enhanced by adsorption of salivary proteins by the streptococci to provide additional adhesin-receptor interactions (Holmes et al. 1995, O'Sullivan et al. 2000). The biofilm can persist as a sessile population of yeasts whose eradication would require removal of the base to which it adheres (Baillie & Douglas 1998).

In healthy individuals the oral recovery of yeasts is about 34% varying from 2% to 71% (Odds 1988b) probably due to the sampling method or site and study population (Arendorf & Walker 1980, Odds 1988b, Brambilla et al. 1992). The numbers of yeasts isolated from the oral cavity of healthy carriers are usually low. In hospitalized patients oral

yeasts are recovered from about 55%, the oral recovery of yeasts varying from 13% to 76% (Odds 1988b).

"The normal oral flora" and "commensals" are terms used for microbes that are almost always present in high numbers in the oral cavity of healthy individuals (Liljemark & Bloomquist 1988, Loesche 1988), meaning that they live in the host, and derive benefit from the host without causing injury (Liljemark & Bloomquist 1988, Asikainen & Chen 1999). Persons infected with disease-causing microbes are called carriers because they do not have symptomatic clinical disease, but can spread an infection to other persons (Salyers & Whitt 1994a). Yeasts can act as opportunistic pathogens, since they, as members of the normal flora, can cause disease in the compromised host (Liljemark & Bloomquist 1988, Cannon & Chaffin 1999). The persistent presence and multiplication of commensal yeast in the oral cavity is called colonization (Salyers & Whitt 1994a, Salyers & Whitt 1994b). The term "infection" is regarded as successful colonization and multiplication by yeasts capable of causing damage to the host in the oral cavity (Liljemark & Bloomquist 1988, Salyers & Whitt 1994b), and an infection that produces symptoms is known as disease (Salyers & Whitt 1994a). Infection can also be regarded as disruption of the host and production of a characteristic group of symptoms (Liljemark & Bloomquist 1988). For disease to occur, yeasts have to adhere and invade host tissues by passing through mucosal surfaces and spreading through the body, penetrating the host's defenses (Salyers & Whitt 1994a, Salyers & Whitt 1994b).

ORAL YEASTS IN HEALTH AND DISEASE

Yeasts are opportunistic pathogens but also regarded as members of the normal oral flora (Arendorf & Walker 1979, Odds 1988b). The dorsum of the tongue is the primary oral reservoir for yeasts (Arendorf & Walker 1980), but yeasts can be also recovered from other oral mucosae, tooth surfaces, and saliva (Arendorf & Walker 1980, Borromeo et al. 1992, Tillonen et al. 1999). In addition to the healthy oral cavity, yeasts have also been found in oral cavities showing dental diseases, such as enamel caries (Hodson & Craig 1972, Sziegoleit et al. 1999), root caries (Lynch & Beighton 1994), and periodontal disease (Slots et al. 1988, Rams & Slots 1991), but the role of yeasts in the etiology of these diseases remains unknown. However, yeasts may play a role in persistent apical periodontitis (Waltimo et al. 1997). High salivary *Candida* counts are related to increased prevalence of caries (Pienihäkkinen et al. 1987), probably because yeasts thrive in the acidic conditions which the caries lesion offers.

The levels of oral yeasts increase along with the presence of fixed or removable orthodontic appliances (Addy et al. 1982) and complete or partial dentures (Budtz-Jörgensen et al. 1975, Berdicevsky et al. 1980, Vandenbussche & Swinne 1984). Studies show that insufficient oral hygiene care and dentures are significant predisposing factors to oral

candidosis (Budtz-Jörgensen 1974). The underlying reasons include environmental changes beneath dentures, such as low pH (Budtz-Jörgensen 1990), which increases the adherence of *Candida* to epithelial and acrylic surfaces (Samaranayake et al. 1980, Samaranayake & MacFarlane 1982). Diets rich in carbohydrates have been suggested to predispose denture wearers to oral candidosis (Samaranayake 1990a). However, recently it has been shown that adding a high amount of refined carbohydrates to the diet of healthy subjects did not increase the number of *C. albicans*-positive subjects (Weig et al. 1999).

Some studies have suggested that tobacco smoking increases oral *Candida* carriage rates (Arendorf & Walker 1980, Kamma et al. 1999, Willis et al. 1999), whereas others have found no association between smoking and yeast carriage (Bastiaan & Reade 1982, Oliver & Shillitoe 1984, Liede et al. 1999). Nevertheless, smoking leads to localized epithelial alterations such as leukoplakic lesions which may enhance colonization of oral *Candida* (Rindum et al. 1994).

C. albicans is the most commonly isolated yeast species in the oral cavity both in health and disease (Odds 1988b). It accounts for about 47% to 75% of the oral yeast isolates, while other medically important yeast pathogens, *Candida tropicalis, Candida parapsilosis, Candida krusei, Candida kefyr, Candida glabrata,* and *Candida guilliermondii,* each represent less than 10% of isolates (Odds 1988b). According to recent reports yeast species other than *C. albicans* cause increasing number of infections (Hazen 1995), including arthritis, osteomyelitis, endocarditis, endopthalmitis, meningitis, and fungemia (Fridkin & Jarvis 1996).

Yeasts, especially *C. albicans* (Slots et al. 1988, Rams & Slots 1991), are recovered not only from the oral mucosae, but are also recovered from periodontal pockets in some patients with adult periodontitis (20%) (Slots et al. 1988, Rams et al. 1990, Slots et al. 1990, Dahlén & Wikström 1995). It is not known whether subgingival *C. albicans* or other yeasts participate in the pathogenesis of destructive periodontal disease, or whether the organism is merely an innocent bystander in periodontal pockets indicating its colonization or even candidal disease at other oral sites. Periodontitis is generally regarded as a mixed bacterial infection (Haffajee & Socransky 1994) for which anaerobic gram-negative bacteria (Slots 1979, Haffajee & Socransky 1994), including *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans,* and additionally *Bacteroides forsythus, Prevotella intermedia* sensu lato, and *Campylobacter rectus,* are the primary etiological agents (Zambon 1996). As stated, *Candida* species and anaerobic periodontal pathogens clearly differ in their growth requirements. Therefore, it can be anticipated that they are not able to thrive for extended periods of time in the same highly specified ecological niche. However, it is possible that ecological changes, e.g. those due to antibiotics (Helovuo 1986, Rams et al. 1990) or

immunocompromising disease or treatment (Slots & Rams 1991) may lead to subgingival yeast overgrowth and even superinfections.

CANDIDOSIS

Candida infections are called candidosis or candidiasis, terms used in the literature as synonyms. The International Society for Human and Animal Mycology (1980) has suggested the term "candidosis", while the Council for International Organizations of Medical Sciences (1982) recommends "candidiasis". The former term will be used in this thesis.

Several factors, such as virulence of the infecting yeast strains, host resistance, and various environmental factors, including sugars and antibacterial drugs (Scully et al. 1994), influence risk for the development of candidosis (Samaranayake 1990b). Despite intense research, no single particular virulence factor of any yeast species has been found to cause candidosis (Cutler 1991, Matthews 1994, Odds 1994). Rather, a combination of virulence mechanisms, such as adhesins, rapid phenotypic switching, hyphal growth, and secretion of hydrolytic enzymes, seems to be responsible for the development of candidosis, since at different stages of candidosis different virulence factors are expressed (Cutler 1991).

An association exists between candidosis and diseases leading to suppression in systemic and local host defenses (Samaranayake 1990a) (Table 1). For instance, in immunocompromising endocrine disorders, including diabetes mellitus, the frequency of oral candidosis increases (Dorocka-Bobkowska et al. 1996, Vitkov et al. 1999). In malignant diseases without therapy the incidence of oral candidosis is greater than in healthy subjects (Scully et al. 1994). Immunosuppressive and corticosteroid therapy lower the host resistance to microbial infections and oral candidosis (Odds 1988c), and the use of broad-spectrum antibiotics suppresses the competing indigenous bacterial flora (Seelig 1966), predisposing the host to over-growth of *Candida* and subsequently to the development of candidosis. In immunocompromised hosts, oral and non-oral candidosis may develop as an endogenous infection (Powderly et al. 1993, Voss et al. 1994) but also can be due to the replacement of original commensal strains by a new invader (Schmid et al. 1992, Powderly et al. 1993). Additionally, altered nutritional status of the host, including iron deficiency (Higgs 1973, Fletcher et al. 1975) and folate deficiency (Samaranayake & MacFarlane 1981), are associated with oral candidosis.

Table 1. Factors, proposed in the literature, predisposing to oral candidosis.

Local factors[a]	Systemic factors
Mucosal barrier	Certain physiological states
Ill-fitting dental appliances	Infancy
Trauma	Old age
Changes in oral environmental conditions	Endocrine disorders
Inadequate home care of oral dental appliances	Diabetes
Endogenous epithelial changes	Hypothyroidism
Atrophy	Nutritional deficiencies
Hyperplasia	Iron deficiency
Dysplasia	Folate deficiency
Saliva	Vitamin B_{12} deficiency
Quantitative changes	Malignancies
Xerostomia	Acute leukemia
Hyposalivation	Agranulocytosis
Qualitative changes	Immune defects
pH	HIV infection
Glucose concentration	Thymic aplasia
Bacteria-yeast coaggregation	Pharmacotherapy
High-carbohydrate diet	Broad-spectrum antibiotics
Tobacco smoking	Corticosteroids
	Cytotoxic drugs

[a] Modified from Samaranayake (1990a), Scully et al. (1994) and Budtz-Jörgensen & Lombardi (1996).

The most frequently used classification to describe various types of oral candidosis is by Lehner (Lehner 1967). The categories in Lehner's classification include acute pseudomembranous candidosis, acute atrophic candidosis, chronic atrophic candidosis, and chronic hyperplastic candidosis. The latter comprises chronic oral candidosis, endocrine candidosis syndrome, chronic localized mucocutaneous candidosis, and chronic diffuse candidosis (Lehner 1964).

Acute pseudomembranous candidosis, commonly referred to as oral thrush (Samaranayake & Yaacob 1990), is characterized by whitish plaques on tongue and oral mucosal surfaces (Korting 1989, Samaranayake & Holmstrup 1989, Holmstrup & Axéll 1990). After removal of plaque, the erythematous mucosal base is exposed. The acute atrophic candidosis, also called midline glossitis or median rhomboid glossitis (Samaranayake & Yaacob 1990), is a possible consequence of the removal of the pseudomembrane (Holmstrup & Axéll 1990). It typically appears on the midline of the dorsal surface of the tongue and is often associated with loss of lingual papillae. In chronic atrophic candidosis, or denture stomatitis (Samaranayake & Yaacob 1990), chronic erythema is usually seen in the palate underlying the denture (Budtz-Jörgensen & Bertram 1970). Denture stomatitis is frequently associated with angular cheilitis, or perléche (Korting 1989, Samaranayake & Yaacob 1990). In chronic hyperplastic candidosis, or *Candida* leukoplakia (Samaranayake & Yaacob 1990), the whitish plaques—which vary from barely palpable patches to firmly

adherent rough plaques on the tongue, palate, or buccal mucosa—are not easily scraped off (Korting 1989, Samaranayake & Holmstrup 1989).

The antifungal agents used currently in the treatment of oral candidosis are azoles: clotrimazole, miconazole, ketonazole, itraconazole, and fluconazole, polyenes: nystatin and amphotericin B, and the disinfectant chlorhexidine gluconate (Budtz-Jörgensen & Lombardi 1996, Cross et al. 2000). The azoles inhibit the cytochrome P-450 enzyme, lanosterol 14α demethylase, in fungal organisms and block steroid synthesis in the fungal cell membrane. This interference leads to increased permeability of the cell membrane (White 1997). The polyenes target ergosterol in the fungal plasma membrane and form pores, leading to increased membrane permeability (White 1997). Chlorhexidine may mainly inhibit the adherence of *Candida* species to oral mucosa and acrylic surfaces (Budtz-Jörgensen & Lombardi 1996).

ACQUISITION OF ORAL YEASTS

Knowledge of the potential sources of yeasts colonizing the oral cavities of infants is limited. The mother's vaginal canal has been suggested as one source of oral yeasts in newborns (Kozinn et al. 1958, Alteras & Aryeli 1980, Baley et al. 1986). Since *C. albicans* is the most common yeast species in the vagina (64-72% of yeast isolates) (Sonck 1978, Goldacre et al. 1981), it is unlikely that infants would commonly acquire other yeast species besides *C. albicans* from their mothers during the delivery. Other possible origins of oral yeasts to the newborn are the hands, skin, mouth, and throat of people taking care of the infant (Pedersen 1969, Sharp et al. 1992, Weems 1992, Sanchez et al. 1993). Only a few studies exist, mainly case-reports, on person-to-person transmission of oral yeasts between family members, such as between husband and wife (Schmid et al. 1990, Mehta et al. 1999), between siblings (Mehta et al. 1999), and between a parent and child (Mehta et al. 1999). Therefore, further studies using larger study populations are needed to determine whether the mother or other care-givers may be major sources of oral yeast infection in early childhood.

C. albicans can be recovered from environmental samples, such as water, soil, and plants, with human or animal sources probably the origin of the organism (Odds 1988b). *C. albicans* survives well on moist surfaces and can be isolated from the toothbrushes of subjects with oral yeasts (Koch & Koch 1981). It may also survive in eye cosmetics (Wilson et al. 1971) and hand cream (France 1968). An endophthalmitis outbreak in drug addicts was thought to result from a contaminated diluent used in injections (Shankland & Richardson 1988). In hospitals, *Candida* species are a frequent finding in air, in foods, and on floors and other surfaces (Odds 1988b). Therefore, a nosocomial source is possible if hospitalized

patients acquire oral yeasts (Schmid et al. 1990, Vazquez et al. 1993, Robert et al. 1995). In immunocompromised HIV-infected couples, both partners have harbored oral yeasts (Sangeorzan et al. 1994) and among 60% (3/5) of these couples the same *C. albicans* strain was recovered from both partners (Sangeorzan et al. 1994). In contrast, systemically healthy partners of subjects with HIV infection did not usually harbor oral yeasts (Sangeorzan et al. 1994).

CANDIDA ALBICANS

Taxonomy

C. albicans belongs to the kingdom Fungi, the division Eumycota (true fungi), the biologically diverse class Deuteromycetes or Fungi Imperfecti and to the genus *Candida* (Carlile & Watkinson 1996). The genus *Candida* comprises more than 150 species (Odds 1987) which are characteristically white asporogenous yeasts able to form pseudohyphae. Species within the genus *Candida* are characterized primarily by colonial morphology, carbon assimilation, and fermentation capabilities (Larone 1995). *Candida* species grow well at 20ºC to 38ºC (Odds 1988a) and within the pH range from 2.5 to 7.5 (Odds 1988a), although a low pH (Arendorf & Walker 1980, Parvinen & Larmas 1981) even below pH 2 (Odds & Abbott 1980) especially favors their proliferation. Candidal cells primarily multiply by budding. Yeast cells are ovoid, 3 x 5 μm in size. *C. albicans* is a simple diploid eukaryote organism lacking a sexual cycle. It grows in two forms, as a yeast (synonyms: blastospore; blastoconidium) and as a hypha (synonym: mycelium). *C. albicans*, but also *Candida dubliniensis*, produces germ tubes and chlamydospores. *C. dubliniensis* has been designated as a separate yeast species, since it comprises a homogeneous genetic cluster, phylogenetically distinct from the other *Candida* species (Sullivan et al. 1995). On the other hand, because of the high DNA homology of *C. albicans* and *C. stellatoidea* (Donnelly et al. 1999), *C. stellatoidea* has been reclassified and moved to the species *C. albicans* as a sucrose-negative variant of *C. albicans* (McCullough et al. 1999a).

Virulence factors

To colonize the oral cavity, *C. albicans* has to adhere to host surfaces (Cannon & Chaffin 1999). *C. albicans* is a component of the adherent biofilm, as are bacteria, such as streptococci and salivary glycoproteins on mucosal and acrylic surfaces (Holmes et al. 1995, Cannon & Chaffin 1999). Adherence to such surfaces as human buccal epithelial cells (Barrett-Bee et al. 1985, Gilfillan et al. 1998) and acrylic surfaces (McCourtie et al. 1986, Nair & Samaranayake 1996) differs among yeast species. During recent years a vast amount of work has been put to clarify the adherence mechanism, which is thought to be a key factor

in the virulence. *C. albicans* possesses multiple adhesins and perhaps more than one adhesin exists that recognizes a host ligand or cell. To date, most adhesins identified are mannoprotein and in adherence, protein and/or carbohydrate portions have been implicated (Cannon & Chaffin 1999). Yeast forms are considered responsible for colonizing epithelia, but the hyphae are regarded as invasive structures of yeasts related to yeast infection (Cutler 1991). Hyphal growth, initiated by germ tube formation (Barnes et al. 1983, Cutler 1991, Kretschmar et al. 1999), increases adherence properties of yeasts (Samaranayake & MacFarlane 1982, Odds 1994). Thigmotropism, contact sensing, may aid hyphae in penetrating tissues (Sherwood et al. 1992) (Table 2).

C. albicans evades host defenses, for instance by modulating phagocytic host defense mechanisms or immune response by means of its surface properties including hydrophobicity and by changing its surface structures with phenotypic switching (Diamond 1993). When nutritionally stressed, *C. albicans* is able to adapt to different host microenvironments by rapid switching of its phenotype (Soll 1992) or by secreting low molecular weight, iron-chelating compounds known as siderophores (Sweet & Douglas 1991b, Howard 1999). Additionally, *C. albicans* and other *Candida* species secrete hydrolases, including proteinases, phospholipases, lipases, hexosaminidase, and phosphomonoesterase (Ruechel 1990, White et al. 1993, Odds 1994, Wu & Samaranayake 1999, Ghannoum 2000) and produce acidic metabolites including short-chain carboxylic acids (Ruechel 1990), and toxic substances, such as carcinogenic nitrosamines (Krogh et al. 1987a). Enzymatic and metabolic activities damage host cells and subsequently may interrupt the host cell functions. Although *C. dubliniensis* is a closely related species to *C. albicans*, little information is available on its virulence.

Table 2. Putative virulence factors of *C. albicans.*

Virulence factor[a]
Adherence
Persorption
Dimorphism
Germ tubes
Rapid switching of expressed phenotype
Thigmotropism
Surface hydrophobicity
Molecular mimicry
Interference with phagocytosis, immune defences and complement
Synergism with certain bacteria
Extracellular hydrolases (proteinases, lipases)
Anaphylatoxins
Killer toxins
Nitrosamines
Acidic metabolites
Growth rate
Undemanding nutrient requirement

[a] Modified from Ruechel (1990), Cutler (1991) and Odds (1994).

Characterization of *C. albicans* isolates

Since *C. albicans* is a member of the normal oral flora (Arendorf & Walker 1979, Odds 1988b) but is also able to cause oral and systemic candidosis, it is possible that the pathogenic potential among *C. albicans* strains varies. Methods have therefore been developed to distinguish among *C. albicans* strains. An optimal method for strain distinction should be (1) capable of discriminating between epidemiologically unrelated strains, (2) be reproducible, (3) not too laborious, (4) able to process a large number of strains, and (5) comparatively inexpensive. Methods described in the literature for differentiating yeast strains will be discussed below.

Phenotyping

Biotyping

Biotyping is based on differences in metabolic properties of yeast isolates. The methods used for biotyping *C. albicans* isolates include a system of nine biochemical assessments based on tests for acid and salt tolerance, proteinase production, resistance to 5-fluorocytosine and safranine, and assimilation of urea, sorbose, citrate, and glycine (Odds & Abbott 1980). Later, a test for boric acid resistance was added to the panel (Odds & Abbott 1983). A widely used commercial test, API ZYM, includes 19 hydrolytic enzyme reactions, and a commercial API 20C system tests for assimilation of 19 different carbohydrates. Additionally, a combination of these systems: API ZYM, API 20C, and resistance to boric acid, has been used for biotyping *C. albicans* isolates (Williamson et al. 1987).

Except for the API ZYM system, the other biotyping methods differentiate well among *C. albicans* isolates (Hunter 1991). The API ZYM system revealed only 4 to 9 *C. albicans* biotypes from 126 to 213 clinical *C. albicans* isolates, whereas the other biotyping methods distinguished 7 to 45 *C. albicans* biotypes from 23 to 130 subjects and isolates (Odds & Abbott 1980, Williamson et al. 1986a, Krogh et al. 1987b, Williamson et al. 1987, Korting et al. 1988, Rams & Slots 1991, Xu & Samaranayake 1995). Although the biotyping method of Odds and Abbott (1980), which includes nine biochemical tests, allows in theory differentiation of *C. albicans* into 512 biotypes, 160 *C. albicans* biotypes were found among more than 700 isolates (Odds et al. 1983a, Odds et al. 1983b). This finding led to a suggestion that all the theoretically possible 512 biotypes may not occur in nature (Odds et al. 1983a, Odds et al. 1983b). Despite the broad variety of biotypes, one to two *C. albicans* biotypes have commonly been predominant (21-75%) among clinical *C. albicans* isolates (Román & Sicilia 1983, Williamson et al. 1986a, Williamson et al. 1986b, Krogh et al. 1987b, Williamson et al. 1987, Korting et al. 1988, Rams & Slots 1991, Xu & Samaranayake 1995).

The distribution of oral *C. albicans* biotypes differs between distant geographical locales (Odds et al. 1983b, Xu & Samaranayake 1995). However, the same *C. albicans* biotypes are found, and even the same *C. albicans* biotypes have been shown to predominate in samples from diverse geographical locations (Williamson et al. 1986a, Williamson et al. 1987, Korting et al. 1988, Asakura et al. 1991, Rams & Slots 1991, Xu & Samaranayake 1995). No data have been published on oral *C. albicans* biotype diversity among Finnish subjects other than that of Study II and of Waltimo (2000) on 37 *C. albicans* isolates from apical periodontitis. Additionally, studies on *C. albicans* biotype diversity in diseased periodontal pockets are limited (Rams & Slots 1991), although other oral and non-oral sites have been studied more intensively (Odds et al. 1983b, Williamson et al. 1987, Xu & Samaranayake 1995).

Within an individual, identical *C. albicans* biotypes have been concurrently isolated from different anatomical sites (Odds & Abbott 1980, Odds et al. 1983a). *C. albicans* strains of the same biotype usually persist within a subject (Odds & Abbott 1980, Odds et al. 1983a). However, when the sampling interval was longer than 15 weeks, both oral and non-oral *C. albicans* biotypes had a tendency to change (Odds et al. 1989).

No consistent association has been found in the literature between certain *C. albicans* biotypes and oral or non-oral candidosis (Odds et al. 1983a, Odds et al. 1983b, Krogh et al. 1987b, Xu & Samaranayake 1995).

Serotyping

Serotyping of micro-organisms is based on detection of the reaction between the antigen and the antibody raised against it. The methods commonly used for serotyping *C. albicans* include Hasenclever tube agglutination (Hasenclever & Mitchell 1961a) and its modification using a slide agglutination test (Drouhet et al. 1975, Stiller et al. 1982). These tests are based on agglutination reactions of *C. albicans* cells and antisera raised in rabbits against *C. albicans* serotype A or B strains (Hasenclever & Mitchell 1961a, Brawner & Cutler 1989). A commercial slide agglutination test uses monospecific antiserum raised in rabbits immunized with extracted polysaccharide antigens of *C. albicans* serotype A cells (Iatron factor 6) (Fukazawa et al. 1968, Shinoda et al. 1981). Additionally, *C. albicans* serotyping has been carried out by indirect immunofluorescence assays using either serotype A-specific rabbit antiserum (Auger et al. 1979) or monoclonal antibodies (H9) against extracted mannans of *C. albicans* serotype A (Miyakawa et al. 1986), and recently, by flow cytometry based on detection of immunocomplexes of the fluorescein-coupled antiserum of Hasenclever or Iatron factor 6 and *C. albicans* cells (Mercure et al. 1996).

The serotyping techniques may give different results for the same *C. albicans* strains (Brawner 1991). Slide agglutination tests using monoclonal antibody (H9) have matched poorly with those using Iatron factor 6, probably because the immunoreagents recognize

different *C. albicans* antigens. Additionally, in the Hasenclever tube agglutination technique, different lots of antisera (produced in different animals) resulted in different serotype results (Brawner 1991). Flow cytometry has been suggested to be more reliable than slide immunofluorescence for serotyping *C. albicans* isolates, since in flow cytometry the difficulty of subjective interpretation of results is eliminated (Mercure et al. 1996).

Hasenclever and Mitchell first demonstrated two serotypes, A and B, among *C. albicans* isolates (Hasenclever & Mitchell 1961a). Serotype differences are based on differences in mannan branches in the cell wall of *C. albicans* (Kobayashi et al. 1992). The antigenic differences result from variations in the bonding positions between mannose residues and the number of residues in mannan side chains (Kobayashi et al. 1992). A third *C. albicans* serotype, serotype C, was proposed in an early study (Müller & Kirchhoff 1969), but it has not been widely accepted among researchers. Due to the current distinction between two *C. albicans* serotypes, serotyping seems to be a rather ineffective tool for epidemiological purposes.

The distribution of the two serotypes of *C. albicans* differs geographically. *C. albicans* serotype A isolates are more frequently found in European countries (Stallybrass 1964, Drouhet et al. 1975) than in North America (Hasenclever & Mitchell 1963, Auger et al. 1979, Stiller et al. 1982). The distribution of *C. albicans* serotypes has been mainly studied in samples originating from non-oral body sites (Hasenclever & Mitchell 1963, Stallybrass 1964, Drouhet et al. 1975, Auger et al. 1979, Stiller et al. 1982). In oral candidosis, *C. albicans* serotype A is most commonly recovered (Martin & Lamb 1982, McMullan-Vogel et al. 1999). On the other hand, researchers agree that the occurrence of *C. albicans* serotype B isolates are associated with declining immune status (Brawner & Cutler 1989, Brawner et al. 1992). No relationship has, however, been found between *C. albicans* serotypes and virulence characteristics (Hasenclever & Mitchell 1961b), except for the results presented in a study by Kwon-Chung et al. (1988). They showed that sucrose-negative variants of *C. albicans* serotype B isolates failed to produce infection in mice, whereas *C. albicans* serotype A caused infections with 80% to 90% mortality. It has been suggested that *C. albicans* serotype A-specific antigens are involved in the adherence of *C. albicans* serotype A isolates to human buccal epithelial cells (Miyakawa et al. 1992). However, no difference has been found between the two *C. albicans* serotypes as to their adherence to buccal epithelial cells (Imbert-Bernard et al. 1994).

Within one individual, the distribution of *C. albicans* serotypes is similar at different anatomical sites (Hasenclever & Mitchell 1963). Additionally, in repeated samplings, *C. albicans* isolates are usually of the same serotype (Hasenclever & Mitchell 1963). However, it is not known whether different *C. albicans* serotypes thrive in particular oral sites such as

periodontal pockets and whether some bacteria enhance colonization of different *C. albicans* serotypes in that ecological niche.

Genotyping

Recent advances in molecular biology have provided powerful tools for strain distinction of *C. albicans* strains. The earliest molecular methods used for fingerprinting *C. albicans* strains were karyotyping, restriction endonuclease analysis (REA), and restriction fragment length polymorphism (RFLP). In karyotyping, very large chromosome-sized DNA molecules can be separated with pulsed-field electrophoresis. REA determines variation in the yeast genomes using various restriction endonucleases to detect differences in restriction endonuclease cutting sites (in DNA sequences). In RFLP, fragments obtained by REA are hybridized with DNA probes. REA generates a fingerprint pattern, with a higher number of genomic restriction fragments than RFLP. However, the greater *C. albicans* genotype discrimination ability of RFLP than of REA among clinical isolates (Diaz-Guerra et al. 1997) is probably due to the less difficult interpretation of the RFLP fingerprints. In RFLP a wide variety of probes, including a ribosomal DNA probe (Magee et al. 1987), a mitochondrial DNA probe (Fox et al. 1989), a *C. albicans*-specific repetitive sequence DNA probe 27A (Scherer & Stevens 1988, Fox et al. 1989), and a DNA midrepeat sequence probe Ca3 (Soll et al. 1987, 1989, 1991, Schmid et al. 1990, 1992, 1993, Hellstein et al. 1993, Schröppel et al. 1994, Lockhart et al. 1995, 1996), have been used for the intraspecies discrimination of *C. albicans.* RFLP analysis, especially with the widely used probe Ca3, has been reproducible and suitable for differentiating *C. albicans* isolates. In arbitrarily primed polymerase chain reaction (AP-PCR) analysis (Welsh & McClelland 1990) (synonym: randomly amplified polymorphic DNA (RAPD) analysis) (Williams et al. 1990), the genomic DNA is used as a template and amplified at a low annealing temperature, with use of a single short primer (9 to 10 bases) of an arbitrary sequence. AP-PCR is faster and technically less demanding to perform than REA or RFLP, and it requires smaller amounts of target DNA. However, the reproducibility of AP-PCR is dependent upon the careful standardization of the PCR conditions, and the discriminatory power is dependent on the primer used and optimization of the assay (Williams et al. 1990, Bostock et al. 1993, Dassanayake & Samaranayake 2000). Although RFLP has been more discriminatory than AP-PCR (Pujol et al. 1997) and REA (Diaz-Guerra et al. 1997) it is time-consuming and laborious and thus not well suitable for routine epidemiological studies.

At the end of the 1980s, the molecular biological techniques REA and RFLP were introduced for genotyping *C. albicans*. Later, in the beginning of the 1990s, AP-PCR was introduced for fingerprinting *C. albicans* isolates. Prior studies, mainly using REA and RFLP, indicate that the genetic diversity of *C. albicans* is high, and that certain *C. albicans*

genotypes are geographically widely distributed (Stevens et al. 1990, McCullough et al. 1999b, Schmid et al. 1999) but also that geographical specificity exists among oral and non-oral *C. albicans* isolates (Schmid et al. 1993, Clemons et al. 1997, McCullough et al. 1999b).

Within a subject, usually the same *C. albicans* genotype (Scherer & Stevens 1987, Fox et al. 1989, Stevens et al. 1990, Whelan et al. 1990) but also different genotypes have been recovered from different body sites (Scherer & Stevens 1988, Soll et al. 1989, Schmid et al. 1990, Xu et al. 1999). A study including four subjects reported a simultaneous recovery of multiple oral *C. albicans* genotypes both from a HIV-negative and a HIV-positive subject (Howell et al. 1996). The same *C. albicans* genotypes usually persist over time in oral and non-oral body sites (Fox et al. 1989, Soll et al. 1989, Whelan et al. 1990).

Similar *C. albicans* genotypes appear in subjects with and without candidosis (Powderly et al. 1992, Hellstein et al. 1993) and from oral candidosis of subjects with and without HIV infection (Whelan et al. 1990, Howell et al. 1996). This is consistent with the suggestion that candidosis is not caused by a unique or particularly virulent *C. albicans* strain but more likely by a defect in host defense mechanisms (Whelan et al. 1990). To date, the majority of studies have focused on studying *C. albicans* isolates from HIV-infected patients. Of interest would be to see whether other immunocompromised patients with candidosis harbor similar oral *C. albicans* genotypes as healthy subjects.

AIMS OF THE STUDY

The aims of the present study were:

- to determine the timing and stability of yeast colonization, and the occurrence of various yeast species in the oral cavity of young children

- to clarify the role of the mother's oral cavity as a possible source of oral yeasts to her child

- to determine the identity and diversity of yeast species recovered from the oral cavity of systemically healthy adults

- to determine the clonal heterogeneity of subgingival *C. albicans* isolates within and between systemically healthy individuals from geographically distant locales

- to determine the relationship between the recovery of *C. albicans* and the simultaneous occurrence of periodontal pathogens

- to study whether differences exist in virulence attributes between *C. dubliniensis* and *C. albicans* and in the distribution of genotypes within each species between healthy individuals and those susceptible to yeast infections

Table 3. Yeast isolates and subjects in Studies I-IV and methods used for culture and characterization of yeast isolates.

Study	Yeast isolates/Subjects[a,b]	Origin of yeast isolates	Methods
I	Oral yeast isolates (N=104; 1-17 isolates/subject) from 80 subjects	Mother-child pairs (N=40) from Finland	Non-selective and selective agar media, including SDA and TSBV CHROMagar AP-PCR (primer OPA-03)
II	Oral yeast isolates (N=362; 4-22 isolates/subject) from - oral mucosa (N=52) - subgingival sites (N=168) - saliva (N=142)	Yeast-positive subjects from Finland (N=29) and the United States (N=19) The subjects exhibited - non-periodontitis (N=15) - adult periodontitis (N=32) - early-onset periodontitis (N=1)	SDA, TSBV CHROMagar Assimilation of carbohydrates AP-PCR (primer OPA-03) REP-PCR (primer $(GACA)_4$)
III	Subgingival *C. albicans* isolates (N=300; 5 isolates/subject)	*C. albicans*-positive routine dental clinic patients from Finland (N=20), the United States (N=20), and Turkey (N=20)	TSBV CHROMagar Serotyping AP-PCR (primer OPE-03)
IV	Oral yeast isolates (N=93; 1-5 isolates/subject) *C. albicans* isolates (N=67) from - cheek mucosa (N=5) - saliva (N=36) - tongue (N=26) *C. dubliniensis* isolates (N=26) from - saliva (N=11) - subgingival sites (N=15)	Yeast-positive subjects (N=40) - *C. albicans*-positive routine dental clinic patients (N=9) and one child - *C. albicans*-positive patients with APECED[c] syndrome (N=21) - *C. dubliniensis*-positive routine dental clinic patients (N=9)[d]	SDA, TSBV CHROMagar AP-PCR (primer OPE-03) Virulence characteristics: - high-frequency phenotypic switching - phospholipase production - proteinase production - siderophore production

[a] A total of 812 yeast isolates from 217 subjects were included in Studies I-IV. Several of the isolates were analyzed in more than one study.
[b] If not otherwise mentioned, the subjects showed no known disorder of the immune system or received no immunosuppressive medications.
[c] autoimmune polyendocrinopathy-candidosis-ectodermal dystrophy
[d] Six patients from Finland and three from the United States.

MATERIAL AND METHODS

SUBJECTS AND YEAST ISOLATES

Detailed descriptions of the material and methods are presented in the original publications.

A total of 812 clinical strains from 217 subjects were included in the Studies I-IV (Table 3). The 13 reference strains are shown in Table 4.

Table 4. Reference strains in Studies I-IV.

Yeast species	Reference strains
C. albicans	NCPF 3179 (serotype A)
C. albicans	CCUG 42415 (serotype B)
C. dubliniensis	CD 36[a]
C. famata	CCUG 662
C. glabrata	NCPF 3309
C. guilliermondii	CCUG 35875
C. intermedia	CCUG 38422
C. krusei	NCPF 3848
C. lusitaniae	NCPF 3516
C. marxianus=C. kefyr	CCUG 34328
C. parapsilosis	NCPF 3872
C. tropicalis	NCPF 3290
S. cerevisiae	Baker's yeast

[a] A gift from D.L. Sullivan, Dublin, Ireland.

Study I was a prospective 22-month follow-up study, part of a comprehensive study on factors affecting the carriage of respiratory pathogens and upper respiratory infections in Finnish children from infancy up to two years of age. Unstimulated salivary samples of 40 children (21 females and 19 males) were collected with a sterile plastic pipette at the ages of 2, 6, 12, 18, and 24 months, and paraffin-stimulated salivary samples were collected from their mothers (mean age 29 years, SD 5.6) at baseline. All salivary samples were cultured for yeasts. Consecutive mother-child pairs participating at every sampling occasion were included.

In Studies II to IV only yeast-positive subjects were included.

Study II used a cross-sectional study design. A total of 362 yeast isolates (4-22 isolates/subject) for the study originated in the oral mucosae (52 isolates), periodontal pockets (168 isolates), and salivary samples (142 isolates) of 48 subjects (mean age 43 years, SD 17 years; 25 women and 23 men) with no known immunosuppressive conditions or medications. Consecutive yeast-positive routine dental clinic patients were included. Twenty-nine of these subjects were Finnish and 19 American. The periodontal status of these subjects included

non-periodontitis (N=15), adult periodontitis (N=32), and early-onset periodontitis (N=1). Except for two siblings, all subjects were unrelated to each other.

Study III was cross-sectional. The material consisted of 300 subgingival *C. albicans* isolates (5 isolates/subject) from 60 *C. albicans*-positive consecutive routine dental clinic patients from Finland (20 subjects; 11 women and 9 men; mean age 54.7 years, SD 10.8 years), the United States (20 subjects; 11 women and 7 men; ages unknown; 2 subjects of unknown gender and age), and Turkey (20 subjects; 12 women and 8 men; mean age 31.8 years, SD 10.8 years). The subjects in Study III differed from those of Study II.

Study IV was cross-sectional. The material, 93 yeast isolates (1-5 isolates/subject) from oral samples of 40 subjects, comprised 67 *C. albicans* isolates recovered from cheek mucosa (5 isolates), saliva (36 isolates), and tongue (26 isolates) and 26 *C. dubliniensis* isolates recovered from saliva (11 isolates) and subgingival sites (15 isolates). Nine consecutive Finnish routine dental clinic patients and one Finnish child were chosen for the study. Inclusion criteria included no known disorder of the immune system (defined as healthy subjects) and *C. albicans*-positive oral samples. All 21 Finnish *C. albicans*-positive patients with immuno-compromising autoimmune polyendocrinopathy-candidosis-ectodermal dystrophy (APECED) syndrome were included. The *C. dubliniensis* isolates (N=26) were from all nine routine dental clinic patients (6 Finnish subjects, 3 American subjects) with this yeast species and with no known disorder of the immune system (defined as healthy).

CULTURE AND IDENTIFICATION OF YEAST SPECIES

The oral yeast samples obtained from the oral cavities of the study subjects were inoculated on several non-selective and selective agar media including Sabouraud dextrose agar with antibiotics (G-penicillin 20 U/ml, streptomycin 25 µg/ml) (Table 3). The samples were cultured in the microbiology laboratories of the Institute of Dentistry, University of Helsinki; School of Dentistry, University of Southern California; the National Public Health Institute, Helsinki; and the Mycology Laboratory, Helsinki University Hospital.

The identification of clinical isolates of *C. albicans, C. dubliniensis, C. glabrata, C. guilliermondii, Candida intermedia, C. krusei, C. lusitaniae, C. parapsilosis,* and *C. tropicalis* was carried out by established methods (Larone 1995, Sullivan et al. 1995) and commercial kits (bioMèrieux Vitek, Lyon, France; CHROMagar, Paris, France; Difco Laboratories, Detroit, MI, USA) presented in more detail in the original publications (Studies I-IV).

For all studies of this thesis, the single-colony yeast subcultures stored in 20% skim milk (Difco Laboratories) at -70ºC were revived by culturing on Sabouraud dextrose agar plates (Sabouraud dextrose, Oxoid, Hampshire, UK) or CHROMagar Candida Medium plates

(CHROMagar, Paris, France). The yeast cultures were incubated in air at 37ºC for 2 days and checked for purity.

PHENOTYPIC CHARACTERIZATION OF THE YEAST ISOLATES

Biotyping

Assimilation of carbohydrates was tested with the API 20C Aux kit (bioMèrieux Vitek, Lyon, France). The reactions were assessed after 24, 48, and 72 hours of incubation at 30ºC according to the manufacturer's instructions. Each yeast isolate was tested one to three times. In repeated testing, the assimilation results obtained with API 20C Aux kit were reproducible. Biotyping is described more in detail in Study II.

Serotyping

C. albicans isolates were serotyped by using a slide agglutination test according to manufacturer's instructions (Candida Check, Iatron Laboratories, Inc., Tokyo, Japan). *C. albicans* serotype A was identified based upon the presence of agglutination, and *C. albicans* serotype B based upon absence of agglutination indicated by the commercial antiserum IF6 included in the kit. Isolates that autoagglutinated in saline after repeated testing were characterized as non-serotypeable. Serotyping is described more in detail in Study III.

Virulence factors

The virulence factors were studied in Study IV.

High-frequency (10^{-2}-10^{-1}) phenotypic switching was determined by modifications of the method of Jones et al. (1994). After incubation on Sabouraud dextrose agar plates, yeast isolates were inoculated on agar plates of the medium of Lee et al. (1975) supplemented with arginine and zinc (Anderson & Soll 1987). The ratio was calculated of the total number of switched colonies to all colonies on the plate (Soll et al. 1987, Study IV). Each isolate was tested once.

Phospholipase production of the yeast isolates was determined according to a modification of the method of Price et al. (1982). After incubation on Sabouraud dextrose agar plates, the yeast isolates were streaked on Sabouraud dextrose agar/Egg yolk plates (Price et al. 1982), and the plates were incubated at 37°C for 48 hours. The phospholipase activity of the isolates was interpreted as positive when a precipitation zone was visible surrounding the growth. The phospholipase production of each isolate was tested twice.

Proteinase production of the yeast isolates was determined according to a modification of the method of Ray and Payne (1990). After incubation on Sabouraud dextrose agar plates, the yeast isolates were streaked on bovine serum albumin agar plates prepared according to Staib (1965). After incubation, the plates were washed, stained, and destained. The proteinase

activity of the isolates was interpreted as positive when a clarification zone was visible around the growth. The proteinase production of each isolate was tested twice.

Siderophore production of the yeast isolates was determined according to a modification of the methods of Schwyn and Neilands (1987) and Alexander and Zuberer (1991). After incubation on Sabouraud dextrose agar plates, the yeast isolates were grown on agar plates supplemented with iron (Sweet & Douglas 1991b). The siderophore production of the isolates was interpreted as positive when a color change from light blue to pink was visible around the growth, and the color of the growth changed from light cream to pink. The siderophore production of each isolate was tested twice.

GENOTYPIC CHARACTERIZATION OF THE YEAST ISOLATES

DNA extraction

A rapid lysate method was developed for DNA extraction by modifying the method of Bollet et al. (1991). Briefly, after incubation of yeast cells in Tris-EDTA (TE) buffer and sodium dodecyl sulfate, the supernatant was discarded and the yeast pellet was heated in a microwave oven and dissolved in TE buffer, and a 1:100 dilution of the cell lysate was used as the DNA template in PCR amplification (Study II).

Genotyping

PCR genotyping of the yeast isolates was carried out with a repetitive sequence primer $(GACA)_4$ (Schönian et al. 1993), a random sequence primer OPA-03 (5'-AGTCAGCCAC-3', Operon Technologies, Alameda, CA, USA), or a random sequence primer OPE-03 (5'-CCAGATGCAC-3', Operon Technologies). The PCR amplifications were performed as described in detail in the original publications (I-IV). Amplification products were analyzed electrophoretically in 1% (OPA-03-PCR, $(GACA)_4$-PCR) and 2% (OPE-03-PCR) agarose gel, stained with ethidium bromide (0.5 μg/ml), visualized under ultraviolet light, and photographed.

STATISTICAL ANALYSES

The statistical significance of differences in the frequency distributions of the classified variables between the study groups was determined by Fisher's exact test. Mann-Whitney's U-test served for comparing the means of the continuous variables with each other. A P value <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

ORAL YEAST SPECIES IN YOUNG CHILDREN

Occurrence

Of the children, 43% (17/40) were yeast-positive at least once during the follow-up period from 2 to 24 months of age. The recovery rate of salivary yeasts from children remained nearly the same at each sampling occasion throughout the follow-up study (18% of children yeast-positive at age 2 months, 13% at 6 months, 20% at 12 months, 20% at 18 months, and 13% at 24 months). Only a few other longitudinal studies on the occurrence of oral yeasts in young children appear in the literature (Pedersen 1969, Russell & Lay 1973). In contrast with the present findings, the recovery of oral *Candida* species from 140 infants increased from seven days (14%) up to one month of age (82%) and then decreased from this peak towards the age of two months (78%), six months (60%), and one year (50%) (Russell & Lay 1973). Similarly, oral yeasts were recovered from 8% of 75 infants at the age of seven days but from 41% at 5 to 12 months of age (Pedersen 1969). Due to the rather low number of children included in the present study, the lower recovery rate of oral yeasts in the present children up to the age of one year as compared with that in other studies is difficult to explain except for chance. Because infants in the present study and in the prior studies (Pedersen 1969, Russell & Lay 1973) were randomly chosen, systemically healthy infants from Finland, Denmark (Pedersen 1969) or England (Russell & Lay 1973), their differences in the health status or geographical background do not clearly explain this difference in yeast recovery rate. However, different sampling methods were used: salivary samples in the present study and oral mucosal swab samples in the other two studies. Nor does inadequacy of the sampling method seem to be the reason for the present low recovery rates. Saliva (30-40%) (Arendorf & Walker 1980, Brambilla et al. 1992), imprint cultures (44-67%) using square foam pads dipped in Sabouraud's broth to quantify yeasts in certain areas of the oral cavity (Arendorf & Walker 1980, Samaranayake et al. 1986b), or rinse cultures (62-64%) (Samaranayake et al. 1986b, Berkowitz et al. 1994) have been shown to be superior to swab samples (17%) (Odds 1988b) for the recovery of oral yeasts from children and adults. Different culture methods, as well may not explain this result. In the present study, oral yeasts were recovered from several non-selective and selective agar media, including Sabouraud dextrose agar with antibiotics (Larone 1995) and TSBV (our unpublished results) which support well the growth of yeasts, whereas in the prior studies yeasts have been recovered from candida agar (Oxoid) after incubation in Sabouraud's broth (Russell & Lay 1973) or from a solid growth medium for yeasts which was not further specified (Pedersen 1969).

The recovery of oral yeasts from these children was associated with use of a pacifier beyond age 12 months, eruption of the first teeth after six months of age, the mother cooling

the child's food by blowing on it, and the mother cleaning the child's pacifier in her own mouth. These results agree with those of prior studies in which recovery of yeasts was associated with use of a pacifier or nursing bottle at night (Sio et al. 1987, Darwazeh & Al-Bashir 1995, Ollila et al. 1997). As suggested by Sio et al. (1987) the habit of pacifier sucking may disturb the ecosystem of the mouth, resulting in increased yeast recovery. Interestingly, the occurrence of the yeasts in these children was not related to respiratory infections or use of antibiotics. This disagrees with another Finnish study on 1-4-year-old children in whom the occurrence of yeasts was associated with antibiotics (Ollila et al. 1997).

C. parapsilosis was recovered from 55% (18/33) and *C. albicans* from 36% (12/33) of the yeast-positive salivary samples of children during the present 22-month follow-up study. A significant difference (Φ^2=0.2549, P=0.009) appeared between the detection rates of *C. parapsilosis* and *C. albicans* beyond six months of age but not when all sampling occasions were included. This finding was interesting, since to our knowledge, only a single earlier study exists reporting a predominance of oral *C. parapsilosis* in young children (Contreras et al. 1994). In that follow-up study on 124 Spanish children followed from 15 days to 16 months of age, *C. parapsilosis* was detected in 52% of all yeast-positive samples. Sampling method seemed to have no effect on recovery rate of *C. parapsilosis,* since the organism predominated both in salivary (Study I) and swab samples (Contreras et al. 1994). However, in other studies on young children up to the age of two years—regardless of whether the study subjects originated in Europe or the United States—*C. albicans* was the most frequently detected oral yeast species (up to 59% of yeast-positive subjects), with *C. parapsilosis* the next in frequency (up to 38% of yeast-positive subjects) (Pedersen 1969, Blaschke-Hellmessen 1970, Kleinegger et al. 1996). This difference in the recovery rates of oral *C. parapsilosis* (Contreras et al. 1994, Study I) and *C. albicans* (Pedersen 1969, Blaschke-Hellmessen 1970, Kleinegger et al. 1996) cannot be explained by differences in age or health status, since the children were of similar ages and with no diagnosed underlying systemic disease.

Stability of colonization

Our results suggest that yeasts are transient colonizers of young children. Several findings support this suggestion: No child among the 17 yeast-positive children was yeast-positive at each of the five sampling occasions. Furthermore, yeasts were recovered at only one sampling occasion from 35% (6/17) of the children. Yeasts were recovered at several sampling occasions from 65% (11/17) of the children. Of these 11 children, six (55%) exhibited different yeast species and five (45%) exhibited yeasts of only one single species during the 22-month follow-up. The present results are in accordance with a previous longitudinal study reporting inconsistent recovery of yeasts from children's oral cavities

(Russell & Lay 1973). The authors suggested that the occurrence of *Candida* in oral samples may have been underestimated, since swab samples were used instead of saliva and mouth rinses. This may not be the case, because our own results obtained by use of salivary samples corroborated.

Similar to *C. albicans, C. parapsilosis* also seemed to be a transient colonizer, and the other yeast species were only infrequently found. *C. albicans* occurred in 55% (6/11) of yeast-positive children who were yeast-positive at multiple sampling occasions (Table 2 from original publication I). In 67% (4/6) of them, *C. parapsilosis* was detected later; 33% (2/6) of the children with two solely *C. albicans*-positive samples were later yeast-negative. *C. parapsilosis* occurred additionally in 45% (5/11) of children with multiple yeast-positive sampling occasions. Of these children, 40% (2/5) harbored in addition to *C. parapsilosis* another yeast species (*C. guilliermondii,* or *C. lusitaniae* and *C. intermedia*) at earlier sampling occasions, and 60% (3/5) of the children with two or three solely *C. parapsilosis*-positive samples were on earlier occasions and/or in between and/or at later sampling occasions yeast-negative.

As previously suggested (Russell & Lay 1973), changes in the prevalence of oral yeasts along with the age of the infant may be linked with simultaneous changes in the rest of the developing oral microbial flora. To our knowledge, there are no studies in the literature on the stability of oral yeast colonization in young children, at the individual level, except for one single study on 21 infants followed from birth to the age of four weeks (Waggoner-Fountain et al. 1996). In accordance with our results, none of those infants harbored yeasts at every one of the four sampling occasions: yeasts occurred in 33% (7/21) of the infants, of whom 71% (5/7) harbored yeasts at multiple (3/4) sampling occasions and 29% (2/7) at one single occasion (Waggoner-Fountain et al. 1996). Multiple oral *C. albicans*-positive samples were found in one infant in addition to multiple rectal *C. albicans*-positive samples. Rectal *C. albicans* isolates were also recovered from two (40%) other infants. Multiple rectal *C. parapsilosis*-positive samples were recovered from 40% (2/5) of the infants. The single non-oral yeast-positive samples of two infants contained *C. albicans* and *C. parapsilosis.*

Yeast transmission from the mother

Saliva, in contrast to bioaerosols, is regarded as the most probable vehicle in the person-to-person transmission of oral microbes (Asikainen & Chen 1999). Oral microbes are likely transmitted via salivary contact from the mother to her child for example when the mother cleans her child's pacifier in her own mouth or tastes the child's food with the child's spoon. Therefore, salivary samples both from children and their mothers were studied to determine whether the mother was a possible source of her child's oral yeasts.

Fifty percent (20/40) of the mothers were yeast-positive, and 90% (18/20) of them harbored exclusively *C. albicans*, 5% (1/20) both *C. albicans* and *Saccharomyces cerevisiae*, and 5% (1/20) exclusively *C. dubliniensis*. Of these yeast-positive mothers, 35% (7/20) had a child with at least one yeast-positive oral sample. Five of these seven mother-child pairs were positive for *C. albicans*, and in three of these five pairs (60%), the AP-PCR profiles of the *C. albicans* isolates were identical, suggesting transmission. Thus, probable transmission of yeasts from the mother to her child via saliva was detected in 15% (3/20) of the yeast-positive mothers and in 8% (3/40) of all the mother-child pairs.

In the present study, a single isolate only was analyzed. However, earlier studies have suggested that one individual usually harbors only one *C. albicans* genotype (Fox et al. 1989, Whelan et al. 1990). Thus, genotyping multiple *C. albicans* isolates from the present mother-child pairs may not have changed the findings regarding identity and non-identity of the strains within mother-child pairs. Information on the vertical transmission of oral yeasts obtained by molecular biological techniques is limited. In agreement with the present results, one study using REA for genotyping *C. albicans* isolates reported that oral or non-oral *C. albicans* can be transmitted from mother to newborn (Waggoner-Fountain et al. 1996). They showed that 24% (5/21) of the mother-child pairs were yeast-positive, of these, 60% (3/5) harbored *C. albicans*, with the oral or rectal *C. albicans* strain being identical within each of the three *C. albicans*-positive pairs. In another study, 60% (3/5) of *C. albicans* positive mother-child pairs harbored an identical oral *C. albicans* isolate, as determined by AP-PCR genotyping (Mehta et al. 1999).

Of our children, 23% (9/40) were yeast-positive despite the mother's oral samples being yeast-negative. Although the oral route seems to be the source of yeasts from mother to child (Pedersen 1969, Waggoner-Fountain et al. 1996, Mehta et al. 1999), *C. albicans* can be also transmitted to the oral cavity of the child from other body sites which harbor *C. albicans*, such as the mother's vagina during delivery (Kozinn et al. 1958). *C. albicans* is also recovered from human skin and hands (Odds 1988b, Strausbaugh et al. 1994). Thus, in the present study, despite *C. albicans*-negative oral samples, the role of the mother as the source of *C. albicans* to her child cannot be fully ruled out.

Because oral yeast colonization in adults seems to be fairly stable (Samaranayake 1990a; own unpublished data), it was presumed that the baseline salivary sample of the present mothers would define their yeast colonization status throughout the 22-month follow-up period. Since only a baseline sample was collected from the mothers, in 57% (4/7) of the yeast-positive mother-child pairs the yeast isolates were not recovered at the same sampling occasion. Four of the seven children from the yeast-positive mother-child pairs were yeast-negative at baseline but later positive. Although the baseline sample of the child was yeast-negative, yeasts may have existed but below the present detection level.

C. parapsilosis was recovered from none of the mothers although it was recovered from 33% (13/40) of the children. The oral cavity of the mother thus did not seem to be the source of *C. parapsilosis* to her child. In contrast to *C. albicans,* the source of *C. parapsilosis* for the child is probably not the vaginal flora of the mother during delivery (Waggoner-Fountain et al. 1996) because *C. parapsilosis* is only a minor finding in the vaginal flora, whereas the most common species is *C. albicans* (Sonck 1978, Sautter & Brown 1980, Goldacre et al. 1981). However, *C. parapsilosis* can be found on human skin surfaces (Weems 1992) and may thus, for instance, be transmitted from breast skin to the child (Weems 1992, Sanchez et al. 1993, Strausbaugh et al. 1994). All the children in the present study were breastfed. On the other hand, *C. parapsilosis* may have also been transmitted from the hands of people caring for the infant (Weems 1992, Sanchez et al. 1993, Strausbaugh et al. 1994). *C. parapsilosis* is the most frequently isolated *Candida* species recovered from the hands of nurses (50%) and other hospital personnel (67%) (Strausbaugh et al. 1994), and *C. parapsilosis* can thus probably be transmitted to the patients via hands of hospital personnel. *C. parapsilosis* has been suggested to be transmitted between neonatal intensive care unit patients via the hands of nurses (Saxen et al. 1995). Sanchez et al. (1993) indicated that among patients who were *C. parapsilosis*-negative before admission to the hospital, the organism was later found in samples taken from the oropharynx, stool, vagina or wounds. The *C. parapsilosis* strains from four of five patients were of the same genotype as the strains cultured from the hands of several staff members (Sanchez et al. 1993). Three genotypes of *C. parapsilosis* were found, of which one was recovered from 80% (4/5) of patients and 75% (3/4) of staff members and from environmental surfaces tested.

IDENTIFICATION, OCCURRENCE AND CHARACTERISTICS OF *C. DUBLINIENSIS*

Isolates included in Study II showed an AP-PCR and a REP-PCR -profile typical for *C. albicans* except for four isolates, which despite biochemical similarity to *C. albicans,* produced an "atypical" PCR-profile. This atypical PCR-profile was similar to the profile of the type strain of *C. dubliniensis* which has recently been separated from *C. albicans* as a novel *Candida* species (Sullivan et al. 1995, Coleman et al. 1998). Thus, these four isolates, presumptively identified as *C. albicans* (Study II), were later during this study (Study IV) designated as *C. dubliniensis*. *C. dubliniensis* was originally reported to produce abundant chlamydospores which are frequently arranged in triplets or contiguous pairs, in contrast to the single chlamydospores of *C. albicans* (Sullivan et al. 1995). However, the present findings (Study I, II, IV) agree with the results of Kirkpatrick et al. (1998) and Schoofs et al. (1997) who observed no difference in the number of chlamydospores, or their formations, between *C. albicans* and *C. dubliniensis* isolates.

In some studies, *C. dubliniensis* has been differentiated on CHROMagar Candida Medium by its dark green colonies as compared to the lighter green colonies of *C. albicans* (Coleman et al. 1997a, Sullivan et al. 1997). Our results are in agreement with the results of other researchers (Schoofs et al. 1997, Tintelnot et al. 2000) who could not differentiate *C. albicans* and *C. dubliniensis* isolates by color of colonies. According to these observations, both species grew as an equal shade of green-colored colonies on CHROMagar Candida Medium plates. The present *C. dubliniensis* isolates did not assimilate xylose (Study II); such non-assimilation is regarded as a typical property of *C. dubliniensis* (Sullivan et al. 1995, Kirkpatrick et al. 1998, Gales et al. 1999, Pincus et al. 1999). Originally, Sullivan et al. (1995) reported that *C. dubliniensis* does not grow at 42ºC when incubated on potato dextrose agar or yeast peptone dextrose broth. All the present *C. dubliniensis* isolates were able to grow at 42ºC on Sabouraud dextrose agar (Study II). It was recently reported that some strains of *C. dubliniensis* were, however, able to grow at 42ºC but not at 45ºC on Sabouraud dextrose agar or on Emmons' modified Sabouraud glucose agar (Kirkpatrick et al. 1998, Pinjon et al. 1998, Jabra-Rizk et al. 1999), but our *C. dubliniensis* isolates were not tested for growth at 45°C.

Genotypic methods have recently provided rapid and reliable tools for the species differentiation between *C. albicans* and *C. dubliniensis* (Sullivan et al. 1995, Coleman et al. 1997a, Pujol et al. 1997, Sullivan & Coleman 1998). In the present studies both the random sequence primers and the repetitive sequence primer easily differentiated *C. dubliniensis* from *C. albicans* (Study I, II, IV).

The majority of *C. dubliniensis* isolates reported in the literature have been recovered from the oral cavity, suggesting that this species may be particularly adapted to oral colonization (Sullivan et al. 1995, Coleman et al. 1997b, Gilfillan et al. 1998). There are, however, studies indicating that it can also be isolated from blood, feces, sputum, and the vaginal flora (Odds et al. 1998, Pfaller et al. 1998, Pinjon et al. 1998). In early studies, *C. dubliniensis* was mainly found in HIV-infected subjects (Sullivan & Coleman 1998). However, the present *C. dubliniensis* isolates were recovered from saliva and subgingival sites of subjects with no known disorder of the immune system. Recently, other investigators have also reported recovery of *C. dubliniensis* from subjects not described as HIV-infected (Moran et al. 1997, Odds et al. 1998).

Since data on *C. dubliniensis* are still limited, the purpose of the present study (Study IV) was to screen for differences between *C. dubliniensis* and *C. albicans* as regards a number of virulence factors in addition to genotype differences, with tests being chosen that produce results that could be interpreted simply as positive or negative. Information from these studies was intended to assist in focusing future studies on certain virulence characteristics.

The *C. dubliniensis* isolates studied here showed phenotypic switching more frequently (70%) than did the *C. albicans* isolates (22%), a finding that suggests that in this

respect *C. dubliniensis* isolates exhibit higher virulence than *C. albicans* isolates. The ability of a microbe to adapt to environmental changes by switching its phenotype is considered an important virulence factor (Soll 1992). These results are interesting since no earlier data seem to exist on switching of *C. dubliniensis*. However, contrary to the general assumption that *C. dubliniensis* may be more virulent than *C. albicans*, one earlier study suggested in a mouse model lower virulence for *C. dubliniensis* (Gilfillan et al. 1998). In a similar way, our findings on production of phospholipase and proteinase, suggest that the *C. dubliniensis* isolates exhibit lower virulence than the *C. albicans* isolates. Our *C. dubliniensis* isolates did not produce phospholipase, and thus phospholipase production was less frequent among the *C. dubliniensis* isolates than among the *C. albicans* (56%) isolates. Only one study was found in which the quantity of phospholipase production seemed to be similar among oral *C. dubliniensis* and *C. albicans* isolates (Bennett et al. 1998). Our study is probably the first to indicate that the *C. dubliniensis* isolates produced proteinase less often (10%) than did the *C. albicans* isolates (50%) but no significant difference appeared in the production of siderophores between the *C. dubliniensis* (100%) and *C. albicans* (81%) isolates. As the number of tested isolates was limited, further studies using methods that allow more detailed analyses are needed to clarify whether switching ability is a prominent virulence factor of *C. dubliniensis*.

INTERINDIVIDUAL HETEROGENEITY OF ORAL YEASTS

Geographical differences

Yeast species

In Study II, 10 random sequence PCR primers were tested for their ability to discriminate within and between different yeast species. The results indicated that AP-PCR amplification with the random sequence primer OPA-03, or PCR with a repetitive sequence primer $(GACA)_4$ generated distinct amplicon patterns for the yeast species *C. albicans, C. dubliniensis, C. famata, C. glabrata, C. guilliermondii, C. intermedia, C. krusei, C. lusitaniae, C. kefyr, C. parapsilosis, C. tropicalis,* and *S. cerevisiae.*

Detection rates for oral *C. albicans* and *C. dubliniensis* were similar among yeast-positive subjects from Finland and the United States (Study II). *C. albicans* isolates were recovered from 93% (27/29) of Finnish subjects and 89% (17/19) of American subjects. *C. dubliniensis* was recovered from 7% (2/29) of Finnish subjects and 11% (2/19) of American subjects. In addition to oral *C. albicans, C. glabrata, C. krusei,* or *S. cerevisiae* were found concurrently in three Finnish subjects. The American subjects, from whom five isolates per subject were analyzed, did not harbor other yeast species concurrently with *C. albicans.* Our finding that *C. dubliniensis* was detected from both Finland and the United States supports the

assumption that *C. dubliniensis* is distributed worldwide (Sullivan et al. 1997, Jabra-Rizk et al. 1999, McCullough et al. 1999b, Polacheck et al. 2000). Moreover, the present results on the infrequent recovery of yeast species other than *C. albicans* from the oral cavity are in concordance with prior studies (Arendorf & Walker 1980, Brambilla et al. 1992).

C. albicans biotypes and serotypes

The *C. albicans* isolates from 44 Finnish and American subjects (Study II) were divided into six biotypes based on carbohydrate assimilation profiles of the API 20C Aux kit. *C. albicans* populations seem to share similarities worldwide as regards biotype distribution. Among the Finnish and American isolates, *C. albicans* biotype 2576174 predominated (79%). This same biotype is common (57-88%) in previous studies (Williamson et al. 1986a, Korting et al. 1988, Asakura et al. 1991, Rams & Slots 1991) from diverse geographical locations, including Germany, Great Britain, Japan, and the United States. Besides the oral cavity, isolates of this biotype have also been recovered from other body sites (Williamson et al. 1986a, Korting et al. 1988, Asakura et al. 1991, Rams & Slots 1991). Furthermore, two other Finnish biotypes of *C. albicans* (2566174 and 2546074), comprising 4% of the present isolates, were found in 9% of the American isolates studied by Rams & Slots (1991). The remaining four yeast isolates, all of which turned out to be *C. dubliniensis*, were of three additional biotypes solely found among *C. dubliniensis* isolates. It appeared that the API 20C Aux kit is unable to identify some *C. albicans* isolates, as also noticed by Aly et al. (1995). One Finnish *C. albicans* and one *C. dubliniensis* isolate remained unidentified by this kit.

C. albicans serotype A was detected significantly more frequently in subjects from Finland (80%) and Turkey (100%) than from the United States (45%) (Study III). Although no other studies were found on the distribution of *C. albicans* serotypes among isolates from Finland or Turkey, the present finding does generally agree with studies in which the occurrence of serotype A has varied from 75% to 94% of isolates recovered from European countries (Stallybrass 1964, Drouhet et al. 1975) and from 51% to 68% of isolates recovered from the United States (Hasenclever & Mitchell 1963, Stiller et al. 1982).

C. albicans genotypes

Since the intraspecies distinction of *C. albicans* in Studies I and II remained rather poor with the primers OPA-03 and $(GACA)_4$ another random sequence primer, OPE-03, was tested which improved the differentiation among *C. albicans* strains and was thus used in Studies III and IV. It could be seen that one of the 25 *C. albicans* genotypes distinguishable among the present 300 isolates from 60 *C. albicans*-positive routine dental clinic patients occurred at a particularly high frequency (50% of yeast-positive subjects) in subjects from

Turkey, whereas another genotype was rather common in subjects from the United States (35% of yeast-positive subjects) (Study III). No previous studies concerned subgingival *C. albicans* genotypes. *C. albicans* strains shared only one single genotype in all three countries and another genotype in both Finland and Turkey. The other 23 *C. albicans* genotypes were single findings.

Differences in geographic distribution of *C. albicans* genotypes have previously been suggested (Schmid et al. 1993, Pfaller et al. 1998, McCullough et al. 1999b). For instance, *C. albicans* isolates from various geographical locations, including Africa, Asia, Australia, Europe, and South America, showed a similar genotype distribution, one distinct from that found in the United States and Israel (McCullough et al. 1999b). Somewhat unexpectedly, 12 *C. albicans* genotypes were distinguished in the present 20 Finnish subjects, but seven from the 20 American and nine from the 20 Turkish subjects. The ethnically homogeneous population of Finland was originally anticipated to harbor relatively few *C. albicans* genotypes, whereas the heterogeneous population of the United States was expected to harbor several. However, based on our results the *C. albicans* population seems to be heterogeneous in each country, not directly dependent upon ethnic factors but rather showing clustering of certain clones in differing geographic areas.

C. albicans isolates in relation to systemic health

Genotypes and virulence factors

AP-PCR, with OPE-03 as a primer, distinguished 15 *C. albicans* genotypes from 21 immunocompromised subjects with APECED and six genotypes from the 10 healthy subjects (Study IV). No significant difference appeared between patients with APECED and healthy subjects in number of genotypes or in occurrence of virulence factors of *C. albicans* isolates (Study IV).

Our findings were in agreement with those of one study in which the diversity of oral *C. albicans* genotypes between HIV-infected and systemically healthy subjects was similar (Xu et al. 1999). However, in an even earlier study, the diversity of oral *C. albicans* genotypes was lower among 11 immunocompromised subjects (AIDS) than in 16 systemically healthy subjects (Schmid et al. 1992). The authors suggested that the original commensal *C. albicans* strains had been replaced with a new one in AIDS patients, because 45% (5/11) of the unrelated patients with AIDS harbored genetically similar strains. The discrepancies between these studies may result from chance, due to the limited number of subjects or to differences between study populations. The present subjects were immunocompromised patients with APECED and chronic oral candidosis; the others were patients with AIDS (Schmid et al. 1992). Genotyping methods also differed between studies, AP-PCR and RFLP, respectively.

Though we conclude, that further studies with a higher number of subjects are still needed to clarify, whether the diversity of *C. albicans* genotypes is decreased in immunocompromised subjects or is related to immune status.

The same *C. albicans* genotypes were recovered from our systemically healthy subjects as from the immunocompromised patients with APECED, a result in agreement with earlier findings in which the distribution of the *C. albicans* genotypes did not differ among immunocompromised patients with AIDS and healthy subjects (Whelan et al. 1990). Candidosis associated with AIDS is probably not caused by an especially virulent strain but is likely a consequence of a defect in the host's defense mechanisms (Whelan et al. 1990). This assumption was supported by the finding that no particular *C. albicans* genotype is associated with HIV-infection. Oral *C. albicans* isolates were suggested to have a common clonal origin in patients with or without HIV infection (Boerlin et al. 1996).

No significant differences were found in high-frequency phenotypic switching (28% vs. 10% of isolates), phospholipase production (52% vs. 60%), proteinase production (48% vs. 60%), or siderophore production (86% vs. 70%) for oral *C. albicans* isolates between our immunocompromised patients with APECED and candidosis and our systemically healthy subjects (Study IV). These results agree with the findings of Hellstein et al. (1993) who showed phenotypic switching among 34% of *C. albicans* isolates obtained from oral candidosis and among 15% of *C. albicans* isolates from healthy subjects. Contrary to the present results, in the study of Wu et al. (1996) all oral *C. albicans* isolates from patients with HIV infection exhibited proteolytic ability, but only 56% of the isolates from HIV-negative subjects. The discrepancies in presented results do not seem to be methodological, since in both studies proteinase production was tested on similar bovine serum albumin agar plates. There are only a few studies reporting on siderophore production of *C. albicans* (Holzberg & Artis 1983, Sweet & Douglas 1991b), and these usually present a limited number of *C. albicans* isolates, with the main focus on determining siderophore types. Of the present 31 *C. albicans* isolates, 81% produced siderophores, which largely agrees with findings that all 12 *C. albicans* strains from subjects with various pathologies and in asymptomatic patients, in regard to candidosis, produced siderophores (Sweet & Douglas 1991a).

INTRAINDIVIDUAL HETEROGENEITY OF ORAL YEASTS

Yeast species

Of subjects with no known immunosuppression, 85% (41/48) harbored only *C. albicans* isolates and 8% (4/48) only *C. dubliniensis* isolates (Study II). In addition to oral *C. albicans,* also *C. glabrata, C. krusei,* or *S. cerevisiae* occurred in 6% (3/48). The novel chromogenic medium (CHROMagar Candida Medium) used in the present Studies I-IV for

presumptive identification of *Candida* species facilitates recovery of different yeast species from mixed cultures. In contrast to studies concentrating on usually only one yeast isolate per subject, we studied 4 to 22 yeast isolates per subject. Our results are in concordance with those of studies reporting yeast species other than *C. albicans* have seldom been recovered from systemically healthy subjects (Arendorf & Walker 1980, Brambilla et al. 1992). Unlike our findings, oral *C. dubliniensis* has been recovered concurrently with other yeast species (Coleman et al. 1997b, Schoofs et al. 1997). Such a discrepancy may be explained by difference in subjects, since our *C. dubliniensis* isolates were from subjects with no known disorder of the immune system, but the prior isolates from HIV-positive subjects and AIDS patients (Coleman et al. 1997b, Schoofs et al. 1997). Immunosuppression may permit colonization of several coexisting yeast species in the oral cavity.

C. albicans serotypes and genotypes

All five subgingival *C. albicans* isolates per subject from 60 routine dental clinic patients were, within each individual, of the same serotype or non-serotypeable, except for two American subjects who had both serotype B and non-serotypeable isolates (Study III). A literature search revealed no report of two concurrent oral *C. albicans* serotypes per individual; on different sampling occasions one single individual may harbor oral or non-oral isolates of one single *C. albicans* serotype (Hasenclever & Mitchell 1963).

With the primer OPE-03, the discriminative power of the AP-PCR method was relatively high, since it distinguished altogether 33 *C. albicans* genotypes among clinical isolates recovered from 91 subjects and one additional genotype in a reference strain (Studies III and IV). Pujol et al. (1997) found the same primer to generate 17 *C. albicans* genotypes among isolates from 22 subjects, again indicating the favorable discriminative capability of the primer OPE-03. A single *C. albicans* genotype was found in each healthy subject from Finland, the United States, and Turkey among the multiple (N=5) *C. albicans* isolates per subject (Study III, IV), except for two subjects from Turkey who each harbored two genotypes, of *C. albicans* serotype A (Study III). This agrees with prior studies including several isolates per subject and indicates that one individual usually harbors a single oral *C. albicans* genotype (Hellstein et al. 1993, Lockhart et al. 1995, Kleinegger et al. 1996). In these studies the subjects were healthy (no evidence of candidosis or HIV-infection). However, in immunocompromised subjects, findings as to the intraindividual diversity of oral yeasts have been contradictory. A single genotype (Miyasaki et al. 1992, Boerlin et al. 1996) or simultaneously occurring multiple genotypes (Anthony et al. 1995, Redding et al. 1997) of *C. albicans* have been recovered among strains from single subjects.

C. dubliniensis genotypes

The AP-PCR technique with the primer OPE-03 distinguished two genotypes among the 27 oral *C. dubliniensis* isolates originating from nine systemically healthy subjects (Study IV). Except for one subject with two concurrent *C. dubliniensis* genotypes, each subject (N=8) harbored only one. The recovery of only two genotypes may result from the limited number of subjects and isolates tested but also from the fact that the primer was originally optimized for *C. albicans*. It is thus too early to draw conclusions about intraspecies diversity of *C. dubliniensis* until further testing is performed with other AP-PCR primers, or other genotyping methods. Studies have focused mainly on detecting *C. dubliniensis* in clinical samples and differentiating it from *C. albicans.* Only recently, the intraspecies diversity of *C. dubliniensis* has been investigated (Diaz-Guerra et al. 1999, Joly et al. 1999). Fifty-seven *C. dubliniensis* isolates, each from a different subject, exhibited distinct RFLP hybridization patterns (N=53) which were further separated into two distinct genetic clusters (Joly et al. 1999). To date, no studies in addition to the present one have focused on the intraindividual diversity of *C. dubliniensis* genotypes.

SUBGINGIVAL *C. ALBICANS* AND OCCURRENCE OF PERIODONTAL PATHOGENS

A few, albeit geographically inconsistent, associations were evident in the comprehensive analyses of the data between detection of *C. albicans* and of periodontal pathogens from the same samples (Study III). Results on total bacterial counts, detection, and proportions of various periodontal pathogens in relation to the presence or absence of *C. albicans* are discussed below.

First, mean subgingival bacterial counts based on colony-forming units (CFU) were similar in *C. albicans*-positive ($1.5x10^8$ CFU, SD $2.7x10^8$) and *C. albicans*-negative ($1.9x10^8$ CFU, SD $3.7x10^8$) subjects.

Second, when the number of periodontopathogenic species: *A. actinomycetemcomitans, P. gingivalis, P. intermedia* sensu lato, *B. forsythus*, *P. micros,* and *C. rectus* in subgingival samples was compared to the simultaneous occurrence of *C. albicans*, the number of detected species was significantly lower among *C. albicans*-positive Finnish and American subjects than among -negative subjects (10 vs. 15, Φ^2=0.070, P=0.031 and 14 vs. 27, Φ^2=0.590, P=0.035, respectively). Interestingly, the recovery of significantly (Φ^2=0.1837, P=0.013) fewer species of periodontal pathogens per sample also concerned samples with *C. albicans* serotype A isolates from Finland and the United States. Nevertheless, these results suggest that *C. albicans,* and especially serotype A isolates, may thrive in healthy periodontal sites or along with only a few species of periodontal pathogens.

Third, among the selected periodontal species, *P. gingivalis* was recovered significantly (Φ^2=0.1064, *P*=0.008) less frequently from *C. albicans*-positive (29%, 5/17) than -negative (67%, 34/51) subjects in Finland. This finding makes sense because of the differing environmental preferences of *P. gingivalis* and *C. albicans*: in diseased subgingival sites, oxygen pressure is low, host-derived proteins are readily available, and carbohydrate nutrients are limited, all of which favors the obligately anaerobic, proteolytic, asaccharolytic *P. gingivalis* but not the aerobic, fermentative *C. albicans.* Additionally, the mean proportion of *A. actinomycetemcomitans* among the flora was significantly (Z=-2.271, *P*=0.023) higher in Finland among *C. albicans*-positive (mean 0.9, SD 0.9) than -negative (mean 0.1, SD 0.1) subjects. This may be expected, since a similar type of subgingival ecology may favor the growth of both organisms, namely the facultatively anaerobic, saccharolytic *A. actinomycetemcomitans* and *C. albicans*. Coaggregation between yeasts and bacteria may be an important factor in the microbial colonization and progression of infections in the oral cavity. Although no studies on coaggregation between oral *C. albicans* and periodontal pathogens were found, it is known that *C. albicans* binds to several dental plaque bacteria including viridans streptococci (Jenkinson et al. 1990, Holmes et al. 1996), *Fusobacterium* (Grimaudo & Nesbitt 1997), and *Actinomyces* species (Grimaudo et al. 1996). The present study is, to the best of our knowledge, the first to address the question of the coexistence of *C. albicans* and periodontal pathogens in subgingival sites. These results have to be considered preliminary findings, but they encourage further studies to investigate environmental factors favorable for subgingival colonization and persistence of *C. albicans.* In the future, *in vitro* studies should show whether certain periodontal bacteria inhibit or favor the growth of yeasts or *vice versa*.

SUMMARY AND CONCLUSIONS

The present study used phenotypic and genotypic methods to determine the stability of oral yeast colonization in young children and to characterize oral yeasts recovered from children and adults. Additionally, an attempt was made to determine the timing of yeast colonization in the oral cavity of infants and to clarify the role of the mother's oral cavity as a possible source of oral yeast infection for the child. The clonal heterogeneity of oral *C. albicans* isolates was determined between and within subjects from geographically distant locales: Finland, the United States, and Turkey. The simultaneous occurrence of *C. albicans* with the periodontal pathogens *A. actinomycetemcomitans, P. gingivalis, P. intermedia* sensu lato, *B. forsythus, P. micros,* and *C. rectus* was additionally studied. Finally, certain virulence attributes were compared between *C. dubliniensis* and *C. albicans* isolates and between *C. albicans* isolates from subjects with or without a compromised immune response.

All clinical yeast isolates (812 isolates from 217 subjects) included were subcultured on CHROMagar Candida Medium plates for tentative identification. Phenotypic characterization of the isolates was carried out with biotyping and serotyping. PCR was chosen as the technique for genotypic characterization of the yeast isolates because of its relative rapidity and easiness to perform, which allows characterization of a large number of isolates. The random sequence primer OPE-03 improved the intraspecies differentiation of *C. albicans* compared to the primers (a random sequence primer OPA-03 and a repetitive sequence primer $(GACA)_4$) used in the first two studies of the thesis, and so was subsequently used in the two later studies. With the primer OPE-03, a total of 33 genotypes were distinguished among 367 clinical *C. albicans* isolates from 91 subjects and two genotypes among 26 clinical *C. dubliniensis* isolates from nine subjects.

Yeasts were found to be transient colonizers in the developing oral flora of young children, as assessed from salivary samples obtained at ages 2, 6, 12, 18, and 24 months. No child was yeast-positive at each of the five sampling occasions. Of the yeast-positive children, 35% harbored yeasts at one sampling occasion, 41% at two sampling occasions, 18% at three, and 6% at four occasions. Yeasts were recovered on at least one occasion from 43% of children by the age of two years. However, only 28% of children were yeast-positive at multiple sampling occasions. The same yeast species was rarely (18%) detected in successive follow-up samples. *C. parapsilosis* was the most common species (76%) followed by *C. albicans* (24%) in samples obtained at ages over 6 months. Of the mothers, 50% harbored salivary yeasts when tested at baseline, and 95% of the yeast-positive mothers had *C. albicans*. Of these *C. albicans*-positive mothers, 26% had a *C. albicans*-positive child, but no child with *C. parapsilosis* had a *C. parapsilosis*-positive mother with oral sample. The AP-PCR genotypes of the *C. albicans* isolates varied between mother-child pairs, but were

identical within 60% of the pairs, suggesting transmission of oral *C. albicans* between mother and child.

C. albicans was the oral yeast species most frequently recovered from adults (93%). Other yeast species alone (7% of subjects) or together with *C. albicans* (6%) were rare findings. Only a single oral *C. albicans* genotype was usually recovered within a subject (97% of subjects).

Geographical differences were evident in the distribution of *C. albicans* serotypes and genotypes. *C. albicans* serotype A was recovered significantly more frequently from the Finnish and Turkish than the American subjects. Additionally, one *C. albicans* genotype occurred with particularly high frequency in subjects from Turkey and another genotype in subjects from the United States, but none in subjects from Finland.

The present study seems to be the first to study serotypes and genotypes of subgingival *C. albicans* isolates. Subgingival ecology did not seem to favor any particular genotypes. Despite comprehensive analyses, only a few associations were found between the recovery of *C. albicans* and of certain periodontal pathogens, and these associations were, geographically inconsistent.

The *C. dubliniensis* isolates exhibited high-frequency phenotypic switching significantly more frequently, but phospholipase and proteinase production less frequently than for the *C. albicans* isolates. However, no significant difference was found in the expression of these virulence factors or in the distribution of genotypes of *C. albicans* isolates between the subjects with APECED, whose immune system is compromised, and systemically healthy subjects.

The main conclusions are that:

- In the developing oral flora of young children yeasts are transient colonizers, *C. parapsilosis* and *C. albicans* being the most frequently occurring species.

- A child's oral *C. albicans* is partly explained by transmission from the mother, whereas the source of *C. parapsilosis* remains to be settled.

- A subject usually harbors only a single genotype and serotype of oral *C. albicans*, which is the most common oral yeast species in healthy adults.

- *C. albicans*, of which different serotypes and genotypes cluster in geographically distant sites, may be coincidentally present in periodontal pockets.

- Differences appear in the virulence determinants between *C. dubliniensis* and *C. albicans* isolates but not in the virulence determinants or the genotype distribution between *C. albicans* isolates recovered from healthy individuals or those susceptible to yeast infections.

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