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The prevalence of selected genes involved in biofilm formation in *Candida albicans* isolated from the oral cavity

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ABSTRACT

Introduction. *C. albicans* genome sequencing enables investigation of the role of particular genes in biofilm formation involving the yeast-like fungi.
Aim. The aim of the study was to determine the genotypes of *C. albicans* isolates on the basis of the presence of the selected genes involved in biofilm formation.
Material and methods. The study material included *C. albicans* strains isolated from the oral cavity of 654 healthy individuals. The strain biofilm-forming capacity was estimated with the MTT assay and menadione. The presence of *HWP1, ALS3, TUP1, NGR1, SAM2* and *CYS3* genes was investigated.
Results. In total, 15 gene combinations were found, including nine gene combinations for strains with a confirmed biofilm-forming capacity, 11 – for the strains without this capacity, and five – independent of biofilm-forming capacity. A combination involving all the genes occurred in 72.5% of all biofilm-forming strains and in 53.8% of all strains that do not form biofilm. Moreover, the genetic material of 14.3% of all strains not involved in biofilm formation did not contain any of the studied genes. For one of the biofilm-species, no analyzed genes were found.
Conclusions.
1. The absence of correlation between gene combinations *HWP1, ALS3, TUP1, NGR1, SAM2* and *CYS3* and biofilm-forming capacity of the studied *C. albicans* strains confirms the multigenetic – and not yet fully known – molecular basis of the formation of this structure. This result corresponds to the data reported by other researchers.
2. Knowledge on the genetic foundations of biofilm formation is still developing and the list of biofilm-related genes has been considerably extended.
3. The absence of correlation between the combinations of investigated genes and the biofilm-forming capacity of the studied *C. albicans* strains confirms a multigenetic, basis of this structure.
4. The research on genes activated or inhibited during biofilm formation is extremely important, because it would enable the development of effective methods to disturb the biofilm forming process at the molecular level. There is a need for such methods in our clinical practice to prevent biofilm formation in the oral cavity.

INTRODUCTION

The basic factor determining pathogenicity of *Candida albicans* strains is their biofilm forming capacity, and thus numerous studies aim at an understanding of the mechanisms that control biofilm formation at the molecular level. Genetic investigations and research into *C. albicans* genome, conducted over many years, made it possible to acquire the detailed knowledge of the mechanism of biofilm development and of the process of obtaining a unique phenotype by biofilm *in vitro* [1,2].

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AIM

The aim of this study was to determine the genotypes of *C. albicans* isolates on the basis of the presence of the selected genes involved in biofilm formation.

MATERIAL AND METHODS

Buccal swab samples were collected from 654 individuals of both sexes and different ages (Table 1).

The study material (buccal swab samples), immediately after sampling, or after placing in transport medium, was inoculated into Sabouraud's medium with chloramphenicol and ChromAgar Candida. The inoculates were incubated at 35°C for 48 hours.

The initial identification of the yeast-like fungi was based on the macroscopic appearance of the colonies on Sabouraud's medium and the growth of coloured colonies on ChromAgar Candida.

The isolates that formed cream-coloured colonies, smooth or with slightly corrugated surface, convex, shiny, smelling of yeast and cream-textured, were used in the further study. Yeast-like fungi were isolated on Sabouraud's medium. The inoculates were incubated at 35°C for 48 to 72 hours. The microscopic examination of Gram-stained samples showed Gram-positive thin-walled, spherical, cylindrical or egg-shaped blastospores, 4-6 µm in diameter.

The identification of the most frequently detected *Candida* species was performed via API 20 C AUX microtest (bioMérieux Polska Sp. z o.o., Poland).

The capacity of the studied *C. albicans* isolates to form biofilm *in vitro* were examined in stationary conditions with the MTT assay with menadione, generally used in screening tests.

The selected genes were identified: *HWP1*, *ALS3*, *TUP1*, *NGR1*, *SAM2*, and *CYS3*, involved in biofilm formation by *C. albicans* strains isolated from oral ontocenosis in healthy individuals from different age groups. The PCR reaction was performed in 15 µl of mixture (Table 2); its composition differed only in primers, according to the gene we looked for.

Table 1. Tested populations

Tested group	Age interval (years)	Number of people
Neonates, infants, nursery children	0-3	102
Kindergarten children	4-6	82
Primary school children	7-14	91
Secondary school youth	15-18	101
University students	19-25	92
Professionally active individuals	26-45	79
Professionally active individuals, pensioners and retired persons	46-65	53
Nursing home clients	≥ 66	54

Table 2. PCR mixture composition

Volume	Reagents
7.5 µl	REDTaq Ready Mix
0.75 µl	Forward primer
0.75 µl	Reverse primer
1 µl	DNA Matrix
5 µl	Water

Primer sequences for the studied genes were followed:

*HWP*1
5'-TCAGTTCCACTCATGCAACCA-3'
5'-AGCACCGAAAGTCAATCTCATGT-3'

ALS3
5'-GTGATGCTGGATCTAACGGTATTG-3'
5'-GTCTTAGTTTTGTCGCGGTTAGG-3'

TUP1
5'-GCTTCAGGTAACCCATTGTTGAT-3'
5'-CTTCGGTTCCCTTTGAGTTTAGG-3'

NRG1
5'-CACCTCACTTGCAACCCC-3'
5'-GCCCTGGAGATGGTCTGA-3'

SAM2
5'-GGTTCCTTGCCATGGTTGAG-3'
5'-TTGTGTCGACTCTTTTTGGGATAA-3'

CYS3
5'-GTGGTATCGAGTCGTTGATCGA-3'
5'-ACCATTGGCTTCTCTTTCTTCCT-3'

The samples were placed in a thermocycler and the following amplification programs were set:

- 95°C for 5 minutes – initial denaturation,
- 30 cycles including the following stages:
  94°C for 1 minute – denaturation,
  60°C for 1 minute – starter annealing,
  72°C for 1 minute – elongation,
- 72°C for 10 minutes – final elongation,
- 4°C for 20 hours, if the amplification was set for the night.

The amplified PCR products were electrophoresed in 2% agarose gel with 20 µl of ethidium bromide, in TBE buffer, at the voltage of 120 mV. In each gel, we also separated 1 kb DNA markers (100 bp DNA Ladder): 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 base pairs. To visualize the PCR product, the gels were placed in a transiluminator and the photographs were archived in the electronic form and as printouts (Fig. 1).

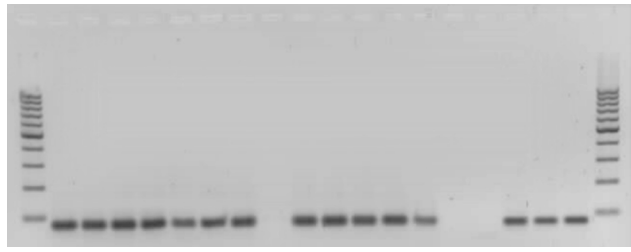

Figure 1. Electropherogram of PCR products stripe pattern for *C. albicans* stripes isolated from the oral ontocenosis of healthy individuals

RESULTS

The oral cavity ontocenosis in the studied population was colonized mainly by the yeast-like fungi of the *C. albicans* genus; they were found in 160 (24.5%) individuals. Using the MTT assay with menadione we showed that 69 (43%) of the *C. albicans* isolates from oral mucosa in the tested population were capable of forming biofilm. We found

15 gene combinations, nine for the strains with a confirmed biofilm formation capacity, and 11 for the non-biofilm forming strains. It must be noted that the biofilm forming strains and the non-biofilm forming ones had five combinations in common.

The most frequent combination, involving all the studied genes: *HWP1*, *ALS3*, *TUP1*, *NRG1*, *SAM2*, *CYS3* (Fig. 2), was found in 50 (72.5%) biofilm forming strains (Table 3) and in 49 (53.8%) non-biofilm forming strains (Table 4). Moreover, gene *SAM2* was relatively frequent; it was found in 8 (11.6%) strains with the confirmed biofilm forming capacity (Table 3) and in 13 (14.3%) of the non-biofilm forming strains (Table 4).

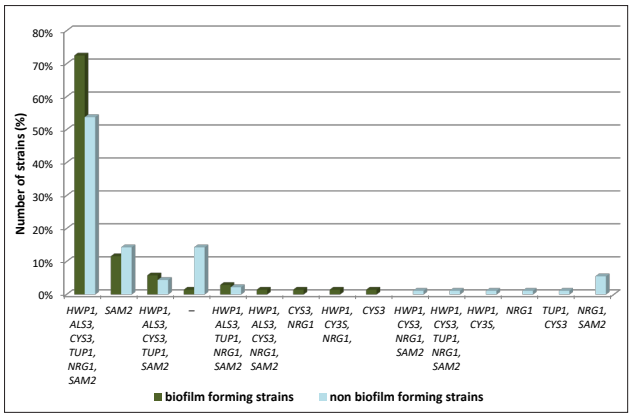

Figure 2. Genes present in *C. albicans* strains isolated from the oral ontocenosis of healthy individuals from different age groups

Table 3. The distribution of gene combinations in *C. albicans* biofilm forming strains isolated from the oral ontocenosis in healthy individuals from different age groups

Gene combination	Number of strains in age groups								Number (percentage) of strains 69 (100%)
	0	1	2	3	4	5	6	7	
HWP1, ALS3, CYS3, TUP1, NRG1, SAM2	3	4	6	7	6	9	6	9	50 (72.5%)
SAM2	1	2	2			3			8 (11.6%)
HWP1, ALS3, CYS3, TUP1, SAM2	1			1	2				4 (5.8%)
HWP1, ALS3, CYS3, NRG1, SAM2	1								1 (1.45%)
-			1						1 (1.45%)
CYS3, NRG1				1					1 (1.45%)
HWP1, CY3S, NRG1,				1					1 (1.45%)
HWP1, ALS3, TUP1, NRG1, SAM2							1	1	2 (2.9%)
CYS3								1	1 (1.45%)

As it is shown in Tables 3 and 4, other combinations were present in very few isolates. It is interesting that the genetic material of 13 (14.3%) of the non-biofilm forming strains and one of the biofilm forming strains did not contain any of the studied genes. It should be emphasized that the combination prevailing in the total studied strains was also most frequent in the strains isolated in different age groups, regardless of their biofilm forming capacity.

The complete list of the genotypes of *C. albicans* strains isolated from the oral ontocenosis of healthy individuals from different age groups, correlated with the yeast-like fungi biofilm forming capacity, can be obtained from the authors.

Table 4. The distribution of gene combinations in *C. albicans* non-biofilm forming strains isolated from the oral ontocenosis in healthy individuals from different age groups

Gene combination	Number of strains in age groups								Number (percentage) of strains 91 (100%)
	0	1	2	3	4	5	6	7	
HWP1, ALS3, CYS3, TUP1, NRG1, SAM2	8	5	7	4	4	4	10	7	49 (53.8%)
SAM2	2	4	1		4	1	1		13 (14.3%)
HWP1, ALS3, CYS3, TUP1, SAM2		1		3					4 (4.4%)
–		3	3	2	3			2	13 (14.3%)
HWP1, ALS3, TUP1, NRG1, SAM2		1					1		2 (2.2%)
HWP1, CYS3, NRG1, SAM2	1								1 (1.1%)
HWP1, CYS3, TUP1, NRG1, SAM2			1						1 (1.1%)
HWP1, CY3S,				1					1 (1.1%)
NRG1				1					1 (1.1%)
TUP1, CYS3					1				1 (1.1%)
NRG1, SAM2					2	3			5 (5.5%)

DISCUSSION

A basic determinant of pathogenicity of *C. albicans* strains is their capacity to form biofilm, and, therefore, the aim of numerous studies was to understand the processes that control biofilm formation at the molecular level. To date, we have discovered a large and still growing list of *C. albicans* genes whose products affect, or may affect, biofilm development *in vivo* and/or *in vitro*. Mutant libraries with deletion of the genes that transcribe individual transcription factors are being developed, as the latter play a principal role in life processes regulation, including biofilm formation control [3]. The analysis of transcription profiles using DNA microarrays points to the presence of genes closely related to biofilm phenotype. All the stages of biofilm formation, including adhesion, are under control of transcription factors, e.g.: Efg1, Cph1, Tec1, Bcr1, and are determined by their activity. A contact of *C. albicans* with a particular surface is a signal activating, among others, MAPK signalling cascade, which initiates transcription factors and expression of a specific set of genes for a given phase of biofilm development. After contact of *C. albicans* with a polystyrene surface, a surprising increase can be observed in the level of transcription of sulphur metabolism genes that encode amino acids: methionine and cysteine, and of *CDR1* and *MDR1* genes that encode the mechanism of active efflux of drugs through cellular membranes. The formation of biofilm structure is conditioned by the expression of many genes. The most important of these include: *BCR1*, *TEC1*, *ALS3*, *HWP1*, *ALS2* (which encode proteins participating in the adhesion process), *EFG1*, *TEC1*, *SUV3*, *NUP8*, *MDS3*, *KEM3*, *MKC1* (which encode proteins participating in the process), or *CHK1*, *YWP1* (which encode proteins responsible for intercellular communication). Genes such as: *NRG1*, *SAM2*, *CYS3* and *TUP1* also take part in biofilm formation [4,5]. Genes *HWP1* and *ALS3*, analysed in this study, encode adhesins present on pseudo-hyphae/hyphae. Genes *NRG1* and *TUP1* are negative regulators of the filamentation process, while genes *SAM2* and *CYS3* are involved in a biosynthesis of sulphur amino acids that

are important for yeast-like fungi cells in mature biofilm [2,6-12].

Five common gene combinations were found, regardless of the biofilm formation capacity of the tested isolates. The combination containing all the mentioned genes occurred most frequently; in addition, SAM2 was found relatively often. It is worth noting that a strain of *C. albicans* that did not contain any of the studied genes was also found.

It is known that adhesion is an indispensable stage of biofilm formation. Herein, Als proteins (Als1-Als 9), i.e. the products of *ALS* genes family, belong among the basic *C. albicans* adhesins [2,13,14]. On the surface of pseudohyphae/hyphae, additional adhesins, among others, the Hwp1 protein encoded by gene *HWP1*, can also be found. Genes *ALS* and *HWP1* are expressed during blastospore-to-pseudo-hypha morphogenesis. However, both adhesins: Als3 and Hwp1, are not always necessary for biofilm formation [2,5,10,15,16].

The literature data and the results of the present study suggest that the process of *C. albicans* adhesion is very complex, and many adhesins and particles participate in co-adhesion with other oral microbes. In addition, it is known that not all biofilm forming strains of *C. albicans* are capable of blastospore-to-psuedo-hyphae/hyphae transformation, despite the fact that *Candida* are dimorphic fungi, their dimorphism being an important factor of their pathogenicity [17,18].

The representative results obtained in the present study (due to a large number of healthy individuals at all the age groups and of both sexes) in the aspect of the participation of the selected genes that condition biofilm formation in *C. albicans* in the context of possible pathological conditions.

CONCLUSIONS

1. The absence of correlation between gene combinations *HWP1*, *ALS3*, *TUP1*, *NGR1*, *SAM2* and *CYS3* and biofilm-forming capacity of the studied *C. albicans* strains confirms the multigenetic – and not yet fully known – molecular basis of the formation of this structure. This result corresponds to the data reported by other researchers.
2. The knowledge on the genetic foundations of biofilm formation is still developing and the list of biofilm-related genes has been considerably extended.
3. The absence of correlation between the combinations of investigated genes and the biofilm-forming capacity of the studied C. albicans strains confirms a multigenetic, basis of this structure.
4. The research on genes activated or inhibited during biofilm formation is extremely important, because it would enable the development of effective methods to disturb the biofilm forming process at the molecular level. There is a need for such methods in our clinical practice to prevent biofilm formation in the oral cavity.

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