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Exopolysaccharides and capsules in human pathogenic *Exophiala* species

Exopolysaccharide und Kapseln bei humanpathogenen *Exophiala*-Arten

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Key words. *Exophiala spinifera*, *Exophiala dermatitidis*, black yeasts, exopolysaccharides, capsules.
Schlüsselwörter. *Exophiala spinifera*, *Exophiala dermatitidis*, Schwarze Hefen, Exopolysaccharide, Kapseln.

Summary. The black yeasts *Exophiala spinifera* and *E. dermatitidis* produce extracellular slimes, which may be either in the form of a well-delimited capsule or of diffusely exuded exopolysaccharides (EPS). The optimal conditions for their production were studied. The presence or absence of polysaccharide material can be used for recognition of the two species. Five-day-old cultures grown on potato glucose agar at 24 °C were observed in India ink, and positive identification for *E. spinifera* was obtained when significant halos were seen around yeast cells. In contrast, *E. dermatitidis* had irregular EPS with a fibrillar substructure made visible by alcian blue staining. Other *Exophiala* species produce insignificant amounts of extracellular mucus or none at all. The diagnostic method is particularly useful with yeast-like primary cultures, which often consist entirely of budding cells and lack the characteristic structures of the filamentous *Exophiala* synanamorph.

Zusammenfassung. Die schwarzen Hefen *Exophiala spinifera* und *E. dermatitidis* produzieren extrazellulären Schleim, der begrenzt kapsulären Ursprungs sein oder von diffus ausgeschiedenen Exopolysacchariden (EPS) stammen könnte. Optimale Produktionsbedingungen dafür wurden untersucht. Die An- bzw. Abwesenheit von Polysaccharidmaterial kann für die Identifizierung der beiden Spezies genutzt werden. Kulturen, die bei 24 °C auf Kartoffel-Glucose-Agar wuchsen, wurden nach 5 Tagen mit Tusche gefärbt. Bei *E. spinifera* bilden sich hierbei signifikante Höfe um die Hefezellen. Im Gegensatz dazu, hat das irreguläre EPS von *E. dermatitidis* eine fibrilläre Substruktur, die mit Alcian-Blau sichtbar wird. Andere *Exophiala*-Spezies produzieren keine oder sehr geringe extrazelluläre Schleime. Diese diagnostische Methode ist besonders nützlich bei Primärkulturen, die häufig nur aus knospenden Zellen bestehen und denen die charakteristische Filamentstruktur des *Exophiala*-Synanamorphen fehlt.

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Introduction

Extracellular polysaccharides (EPS) of black yeasts in the ascomycete order Dothideales have received special attention, particularly in the genus *Aureobasidium*. *Aureobasidium pullulans* (De Bary) Arn. is of practical interest, since its EPS has been widely employed in biotechnology and the food industry [1]. *Aureobasidium* and related genera are plant-associated fungi which are extremely rarely involved in human disease [2]. For this reason black yeast EPS has never been regarded as a virulence factor. However, some black yeasts belonging to the order *Chaetothyriales* also produce extracellular slimes and are frequent agents of mycoses in immunocompromised or debilitated patients, as well as in otherwise

healthy hosts. Most of these infections are superficial, but in some species they may be devastating and fatal. In the genus *Exophiala*, capsular material was reported around very young cells of *Exophiala spinifera* (Nielsen & Conant) McGinnis and *Exophiala jeanselmei* (Langer.) McGinnis & Padhye [3; identity unconfirmed] and acid mucopolysaccharides were observed around yeast cells of *Exophiala dermatitidis* (Kano) de Hoog [4]. It is remarkable that the polysaccharides are specifically associated with the most virulent species of the genus, and hence a role in pathogenicity towards humans may be surmised. Nishimura and Miyaji [4] supposed a role in the interaction between yeast cells and mononuclear neutrophils in mice.

The present article focuses on capsular or EPS in a wider array of *Exophiala* species. The polysaccharide-like compounds may be characteristic for the species, but their presence shows infraspecific variation. Optimal conditions for the production of extracellular material are established in an attempt to determine their diagnostic value. A protocol for their use in identification of *E. spinifera* and *E. dermatitidis* is provided.

Materials and methods

Strains and culture conditions

The strains studied are listed in Table 1. They were maintained on potato glucose agar (PDA) slants. Inoculum grown for 7 days on PDA at 24 °C was transferred to PDA, Sabouraud glucose agar (SGA) and Czapek agar (CzA) amended with different concentrations of carbon and nitrogen sources. The formulations used included the following variations in concentration of these components:
$NaNO_3$ 3 g l^{-1}, sucrose 30 g l^{-1} (standard CzA): CzA30.
$NaNO_3$ 3 g l^{-1}, sucrose 20 g l^{-1}: CzA20.
$(NH_4)_2SO_4$ 2.25 g l^{-1}, sucrose 20 g l^{-1}: CzNHA20.
$(NH_4)_2SO_4$ 2.25 g l^{-1}, sucrose 30 g l^{-1}: CzNHA30.

In media containing 20 g l^{-1} sucrose the C/N ratio is maintained at 17, whereas in media with 30 g l^{-1} sucrose, this ratio is 25. Cultures were incubated at 15, 24, 37 and 40 °C. At 40 °C, only *E. dermatitidis* showed good growth.

Seven-day-old cultures on PDA at 24 °C were used to inoculate tubes with 5 ml of liquid potato glucose (PDL) and the liquid Czapek (CzL) variants CzL20, CzL30, CzNHL20 and CzNHL30, as well as to 250 ml flasks containing 75 ml liquid medium PDL and CzNHL30. The cultures were incubated at 15, 24 and 37 °C. All liquid media were shaken at 200 r.p.m.

Aureobasidium pullulans var. *pullulans*, CBS 584.75, which is known to synthesize the exopolysaccharide pullulan and *A. pullulans* var. *aubasidani* Yurlova, CBS 100524, which is known to synthesize the exopolysaccharide aubasidan [5] were used as controls.

Capsular and EPS observation

Production of extracellular material was investigated regularly during 2–7 days of growth. Positive staining was carried out with alcian blue (Sigma, Zwÿndrecht, The Netherlands), a polyvalent, copper-containing phthalocyanine basic dye, dissolved in

Table 1. List of strains examined

Strain no.	Species name	Authenticity	Source
CBS 207.35	*Exophiala dermatitidis*	T	Skin lesion
CBS 153.90	*Exophiala dermatitidis*		Skin lesion
CBS 109136	*Exophiala dermatitidis*		Steam bath
CBS 109139	*Exophiala dermatitidis*		Steam bath
CBS 109140	*Exophiala dermatitidis*		Finnish sauna
CBS 109143	*Exophiala dermatitidis*		Hall sauna
CBS 899.68	*Exophiala spinifera*	T	Disseminated infection
CBS 670.76	*Exophiala spinifera*		Nest of *Anumbis anumbi*
CBS 671.76	*Exophiala spinifera*		Nest of *Anumbis anumbi*
CBS 101537	*Exophiala spinifera*		Rotten cactus
CBS 131.88	*Exophiala phaeomuriformis*	T	Skin lesion
CBS 109813	*Exophiala phaeomuriformis*		Plastic water container
CBS 584.75	*Aureobasidium pullulans* var. *pullulans*	NT	Fruit of *Vitis vinifera*
CBS 100524 = VKPM F-448	*Aureobasidium pullulans* var. *aubasidani*	T	*Betula*, slime flux
CBS 667.76	*Exophiala jeanselmei*		Rotten wood

T, type culture; NT, neotype culture; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; VKPM, All-Russian Collection of Industrial Microorganisms, Moscow, Russian Federation.

3% acetic acid. The pH of the solution was adjusted to 1 or 2.5 by glacial acetic acid. Another stain used was mucicarmine (Sigma). Microscopic measurements of capsular sizes were carried out, based on negative staining in India ink. All tests were performed three times in duplicate. The numerical values are the means of at least 10 different determinations.

Results

In all strains of *Exophiala spinifera* grown on all solid and liquid media at 24 and 37 °C, capsules with sharp, regular margins were present around almost all yeast cells. The capsulated cells were visible in India ink (Fig. 1–4), as well as in alcian blue and mucicarmine preparations. Staining of the capsule with alcian blue was intense, evenly bright blue, more intense than the cells themselves and therefore clearly distinguishable. The pink staining with mucicarmine was less intense. No substructure was revealed in the capsules stained with alcian blue or mucicarmine. Capsular size was maximal (up to 4 μm) on solid PDA at 24 °C, although considerable variation was noted between strains (Table 2). Capsules were somewhat thicker in shaken liquid versions of the same media. Little difference was observed when sucrose concentrations in Czapek media were altered. In contrast, the use of ammonium as an N-source led to a nearly threefold reduction of capsular sizes ($P < 0.05$). Capsules were generally small in cultures grown at 37 °C, particularly with solid medium. The capsular size was not significantly influenced by the C/N ratio (Table 2).

Some of the cells in very young colonies (1–2 days) of *Aureobasidium pullulans* produced capsular material. After more than 3 days the extracellular material had irregular and diffuse margins and often extended around several adjacent yeast cells. With alcian blue or mucicarmine staining a fibrillar substructure was revealed. The same substructure was observed in *Exophiala dermatitidis* when EPS was stained with alcian blue or mucicarmine. With alcian blue, light blue-stained material was seen to coalesce and often extended to include several yeast cells. Similar observations were made using mucicarmine. The optimal temperature for EPS production was 24 °C (Table 3). The C/N ratio in the modified Czapek agars and liquid media did not influence the production of EPS in these species.

Exophiala dermatitidis strains CBS 109136 and CBS 109139, both isolated from a steam bath, had a slimy phenotype and were confirmed to produce EPS (Fig. 5), whereas CBS 109140 and CBS 109143, from the adjacent hall of the same bathing facility [6] and having the same genotype [7], were dry and lacked EPS.

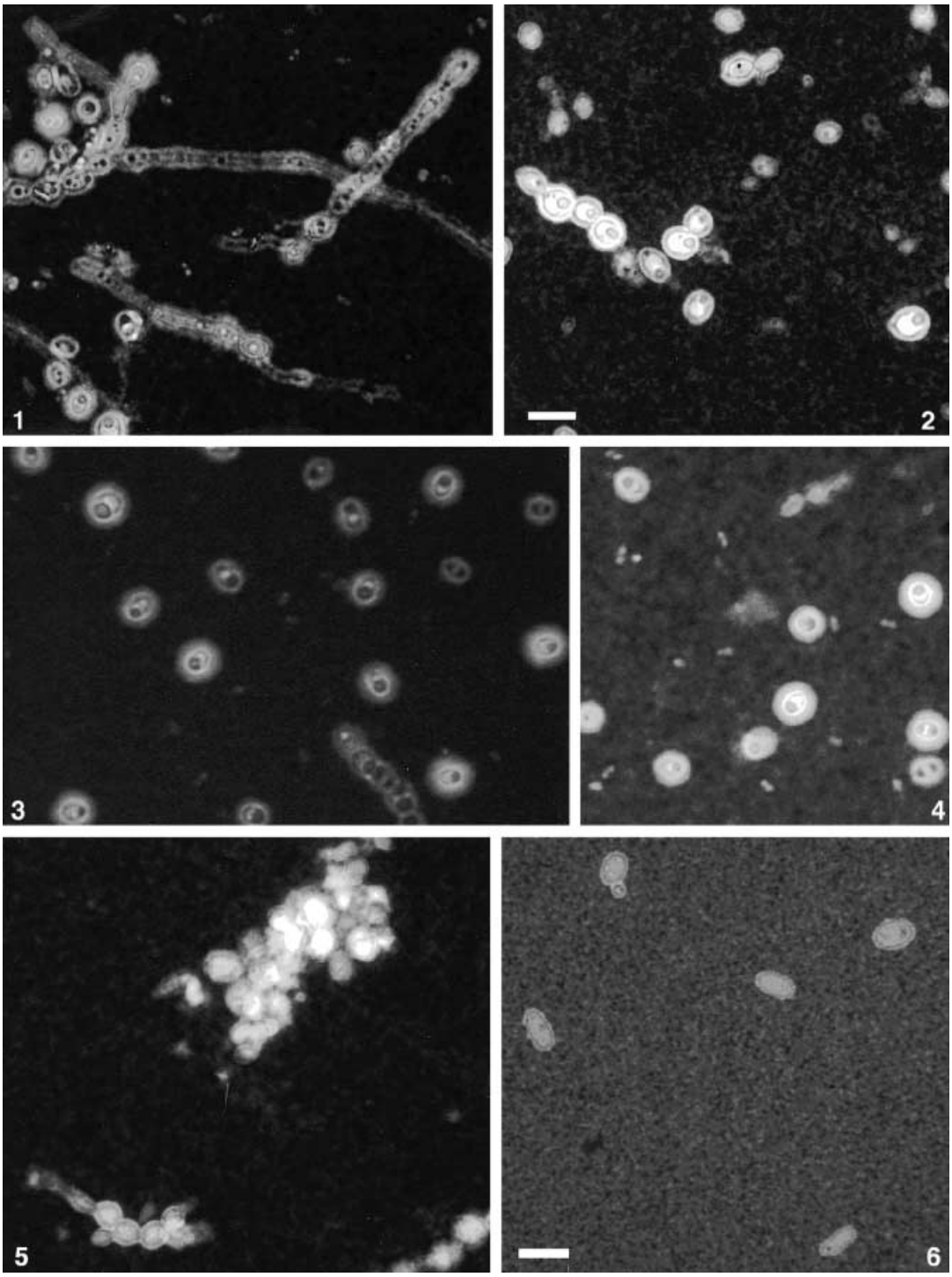
A meristematic strain of *Exophiala phaeomuriformis* (Matsumoto *et al.*) Matos *et al.* (= *Sarcinomyces phaeomuriformis* Matsumoto *et al.*), CBS 131.88, did not produce EPS or capsular material under any of the conditions tested. However, the conspecific strain CBS 109813 with a yeast phenotype, produced some EPS on PDA and SGA (Fig. 6). The material was fibrillar when seen in alcian blue and mucicarmine staining. The optimal temperature for production was 24 °C (Table 3).

Discussion

The irregular extracellular polysaccharide of *Aureobasidium pullulans* resembles that of *Exophiala dermatitidis* in all respects, with alcian blue- or mucicarmine-stained material revealing a fibrillar substructure as a main characteristic. In contrast, *E. spinifera* had regular, sharply defined halos around single cells in India ink and lacked any substructure in alcian blue or mucicarmine. There is a significant morphological difference between the well-defined capsular material of *E. spinifera* on the one hand, and the diffuse EPS of *A. pullulans* and *E. dermatitidis* on the other. It is remarkable that two different types of mucus are produced within the genus *Exophiala*.

The quantity of both types of polysaccharide is influenced by the same set of key factors in all species. The optimal temperature was 24 °C, whereas the largest amounts were formed on the natural medium PDA. On artificial media the production was consistently lower, and in *E. phaeomuriformis* it fell below the detection limit. Extracellular pullulan production in *Aureobasidium* is known to be affected by both type and concentration of nitrogen sources in the growth medium, and on artificial media is optimal with ammonium sulphate [5, 8, 9]. Sodium nitrate is optimal for aubasidan production in *A. pullulans* var. *aubasidani* [5], as well as for the as yet unidentified compound produced by *E. spinifera*.

The presence of capsules or diffuse EPS in *E. spinifera* and *E. dermatitidis* was consistent among strains, although some variation was noted in the amount produced. The quantity produced in early growth accounts for the slimy nature of young colonies of nearly all strains of both species. Matos *et al.* [6] isolated *E. dermatitidis* strains of uniform genotype from a single bathing facility [7] and found that these strains had a lower maximum growth temperature. There are also differences in viscosity [N. A. Yurlova, unpublished data]. The dry phenotype found in some of these strains correlated with the ability to grow at 42 °C and a lack of EPS. The loss of EPS in *E. dermatitidis* is



Figures 1–4. *Exophiala spinifera*, CBS 899.68 on PDA; India ink preparation. Capsules visible around yeast cells and hyphal elements. Bars represent 10 μm.
Figure 5. *Exophiala dermatitidis*, CBS 109139 on PDA; India ink preparation. Coalescent capsules around clusters of cells. Bars represent 10 μm.
Figure 6. *Exophiala phaeomuriformis*, CBS 109813 on PDA; India ink preparation. Capsules nearly absent. Bars represent 10 μm.

therefore associated with decreased thermotolerance. Since thermotolerance is a condition for pathogenicity, a role for EPS in virulence in *Exophiala* may be surmised.

Low quantities of EPS were observed in the yeast phenotype of *E. phaeomuriformis*. This species is phylogenetically related to *E. dermatitidis*; it has some striking physiological properties in common

Table 2. Radius of capsular material produced by *E. spinifera* after 5 days cultivation

Medium	Temperature	Maximum capsular size in µm			
		Strains			
		CBS 899.68	CBS 670.76	CBS 671.76	CBS 101537
PDA	24	4.0	3.6	3.6	2.0
	37	0.8	2.4	2.0	0.8
CzA20	24	2.2	2.4	2.0	1.0
	37	0.4	0.8	1.2	0.4
CzA30	24	2.2	2.4	1.2	1.2
	37	0.4	0.8	0.8	0.4
CzNHA20	24	0.6	0.8	0.6	0.4
	37	0.4	0.4	0.4	0.3
CzNHA30	24	0.8	0.8	0.6	0.4
	37	0.4	0.4	0.4	0.2
CzL20	24	2.8	2.2	2.0	1.6
	37	1.6	1.0	1.2	0.6
CzL30	24	2.8	2.2	2.0	1.6
	37	1.6	1.0	1.2	0.6
CzNHL20	24	1.0	0.4	1.0	0.4
	37	0.8	0.2	0.4	0.2
CzNHL30	24	1.2	0.8	1.2	0.4
	37	0.8	0.6	0.6	0.2

For abbreviations of media, see section Materials and methods.

Table 3. Extracellular polysaccharides in *E. dermatitidis and E. phaeomuriformis* after 5 days cultivation

Medium	Temperature	Extracellular polysaccharides							
		E. dermatitidis						*E. phaeomuriformis*	
		CBS 207.35	CBS 153.90	CBS 109136	CBS 109139	CBS 109140	CBS 109143	CBS 131.88	CBS 109813
SGA	15	+	+ +	+ + +	+ +	–	–	–	+
	24	+ +	+ +	+ + +	+ +	–	–	–	+ +
	37	w	+	+ +	+	–	–	–	w
PDA	15	+	+ +	+ + +	+ +	–	–	–	+
	24	+ +	+ +	+ + +	+ +	–	–	–	+ +
	37	w	+	+ +	+	–	–	–	w
CzNHA20	15	–	w	+	+	–	–	–	–
	24	–	w	+	+	–	–	–	–
	37	–	–	–	–	–	–	–	–
CzNHA30	15	–	w	+	+	–	–	–	–
	24	–	w	+	+	–	–	–	–
	37	–	–	–	–	–	–	–	–
CzNHL20 200 r.p.m.	15	–	–	w	w	–	–	–	–
	24	–	–	+	+	–	–	–	–
	37	–	–	–	–	–	–	–	–
CzNHL30 200 r.p.m.	15	–	–	w	w	–	–	–	–
	24	–	–	+	+	–	–	–	–
	37	–	–	–	–	–	–	–	–

–, negative; w, weak; +, detectable; + +, clearly positive; + + +, highly productive, with slimy cultural phenotype. For abbreviations of media, see section Materials and methods.

with that species [10]. Detectable extracellular material is not known to be produced by any of the remaining *Exophiala* species. Thus, this character can be used for phenotypic recognition of *E. spinifera* and *E. dermatitidis*, which are among the clinically most relevant species of the genus [10]. Rapid recognition of these species can be accomplished using 5-day-old cultures grown on

Table 4. Summary of EPS in 5-day-old cultures of black yeasts grown on PDA at 24 °C

	Defined capsule	Diffuse EPS
Aureobasidium pullulans	–	+
Exophiala dermatitidis	–	+
E. jeanselmei	–	–
E. phaeomuriformis	–	w
E. spinifera	+	–

–, negative; w, weak; +, detectable.

PDA at 24 °C. Yeast cells are mounted and examined in India ink and positive identification is obtained when significant halos are seen around yeast cells. The two species can subsequently be distinguished by revealing the fibrillar substructure of *E. dermatitidis* EPS by alcian blue staining (Table 4). This method is quick, simple and cheap, and is particularly useful in that it can be carried out using only the yeast phase primary cultures without requiring the extended incubation often needed to induce the formation of the characteristic structures of the filamentous *Exophiala* synanamorph.

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