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Antifungal activity of *Bullera alba* (Hanna) Derx

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A strain of *Bullera alba* that secretes a killer toxin inhibitory (at pH values ranging from 3 to 7) to many ascomycetous and basidiomycetous yeast-like fungi was discovered. Its killer phenotype was incurable. The toxin was relatively thermostable and resistant to many proteases, and it was identified as a microcin. It inhibited the growth of some pathogenic yeasts and was the most active against *Cryptococcus neoformans*.

Key Words——*Bullera alba*; killer toxin; microcin.

Production of antagonistic substances is a commonplace phenomenon among microorganisms. About 30 yr ago it was discovered (Makower and Bevan, 1963) that unicellular fungi (yeasts) also secrete antibiotic compounds, which are proteinaceous in nature and possess antifungal action. The antagonistic interactions that cause secretion of these toxins are known now as the killer phenomenon in yeasts (Young, 1987). At present, killer activity has been found in almost 100 species of both ascomycetous and basidiomycetous yeasts, and their number increases every year.

The production of proteinaceous substances that are toxic to related organisms, which is associated with specific immunity, is not unique to yeasts but it is known in smut fungi (Koltin, 1988), slime molds (Mizutani et al., 1990), paramecia (Quackenbush, 1988) and bacteria (Konisky, 1982). Like bacteria that produce bacteriocins and microcins (Baquero and Moreno, 1984), yeasts also produce two types of so-called killer toxins: mycocins, which are (glyco)protein molecules active against taxonomically related organisms (Golubev and Boekhout, 1995); and microcins, which are (glyco)oligopeptides possessing a much broader range of action (Golubev and Shabalin, 1994).

In the course of screening of *Bullera* species for killer activity, we discovered a *B. alba* strain producing toxin that was identified as a microcin. To our knowledge, this is the first report on microcin production by a ballistosporous yeast.

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Materials and Methods

Yeast strains The strains studied (most of them type strains of species) were obtained from the Japan Collection of Microorganisms, the Russian Collection of Microorganisms, and the culture collection of the Dept. Microbiology of Meiji College of Pharmacy.

Medium The assay medium was 0.5% glucose-0.25% peptone-0.2% yeast extract-5% glycerol-2% agar with 0.05 M citrate-phosphate buffer within the pH range 3–8 (at 0.5 intervals). The same medium without agar and glycerol was used for production of toxin-containing culture filtrate.

Bioassay conditions A 0.05-ml portion of an aqueous suspension of 10^6 cells ml^{-1} of the strains examined was put on the surface of the agar medium and throughly spread with a spreader, then a loopful of killer culture was streaked on top. The plates were incubated at 17°C or 8°C until growth of the lawn strain appeared. The strain was recorded as sensitive if a clear inhibition zone of 2–3 mm or more in width developed on the background lawn around the killer strain, and as resistant if the inhibition was absent. Sensitivity was interpreted as weak if the inhibition zone was not more than 0.5–1 mm in width and became overgrown during further incubation.

Characterization of killer toxin After 2 wk of incubation without shaking at 17°C, cells of the killer strain were separated by centrifugation (3,000×*g*, 30 min) and culture supernatant was filtered through glass microfibre filter GF/A (Whatman). This preparation was used to estimate the resistance of the killer toxin to elevated temperature and to proteases, and its binding ability to β-glucan, mannan and chitin (Sigma) by the agar well method. To study killing effect, the culture filtrate was freeze-dried and concentrated by reconstitution in a reduced

volume of distilled water. To detect diffusion of the toxin through moleculaporous dialysis membranes (Spectrum, USA), the killer strain was grown on agar plates covered with these membranes. After 1 wk of incubation (17°C), the membranes and killer cultures on them were removed, and a sensitive strain was inoculated on the agar plate.

Plasmid curing Curing experiments were performed with cycloheximide (0.01%, 17°C) or incubation at 27°C, which is close to the maximal temperature for growth of the killer strain.

Results

Eighteen strains of *Bullera alba* (Hanna) Derx (including 2 strains of *Bulleromyces albus* Boekhout et Fonseca) isolated in Canada, USA, Russia, Japan, New Zealand and Australia were examined for killer activity at pH 4.0 and 5.0. They were tested against *B. albus* VKM Y-2141, *Cryptococcus aerius* (Saito) Nannizzi VKM Y-1540, *Cr. podzolicus* (Babjeva et Reshetova) Golubev VKM Y-2247 and *Cr. terreus* di Menna VKM Y-2253. No activity was found in 17 strains. Growth inhibition activity against all the target strains was found in strain JCM 7495 (=VKM Y-2829), which had been isolated by the ballistospore-fall method from a leaf of *Nothofagus gunnii* (J. D. Hook.) Oersted (Mt Field National Park, Tasmania) in 1987.

Its activity was expressed within the pH range of 3–7. Very weak inhibition of highly sensitive yeasts only was observed at pH 7.0, and no activity at pH 8.0. The broadest inhibition zones developed at pH 4.0 or 4.5 depending on the sensitive strain used. They were wider at incubation temperatures of 8°C and 17°C than at 25°C. The presence of glycerol in the medium also led to broader inhibition zones.

The factor secreted by the strain JCM 7495 has fungicidal action against sensitive yeasts (Fig. 1). It diffused through dialysis membranes with a molecular weight cut-off of 6–8 kDa but not with one of 3.5 kDa.

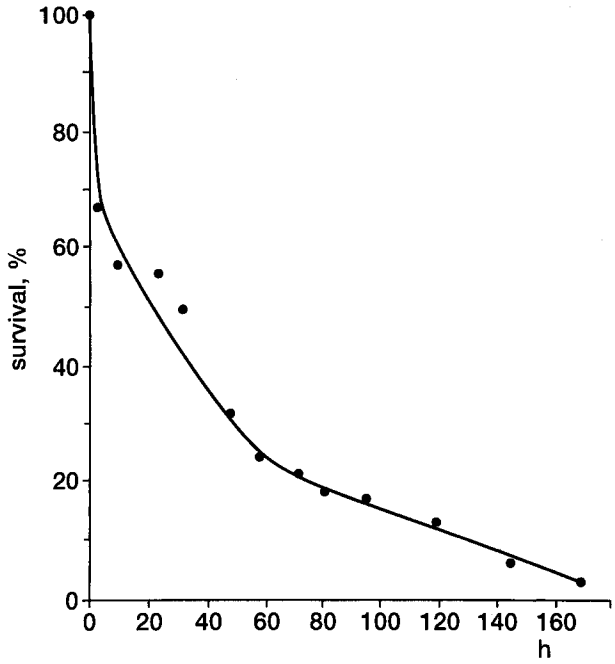

Fig. 1. Kiling activity of the *Bullera alba* JCM 7495 toxin against *Cryptococcus terreus* VKM Y-2253.
The test strain was incubated at 17°C in 2 ml of concentrated (approx. 8x strength) toxin-containing culture filtrate, pH 4.5, with an initial concentration of 2×10^3 cells ml^{-1}. Viability was measured after plating on malt agar, and each point represents the mean of three determinations. Cell viability was calculated as the percent reduction in colony-forming units compared with the control (no microcin).

Table 1. Killing pattern of the *Bullera alba* microcin among basidiomycetes.

Genera (number of species and strains examined)	Number of sensitive species	strains	Genera (number of species and strains examined)	Number of sensitive species	strains
Bensingtonia (10, 10)	3	3	*Rhodosporidium* (7, 9)	4	6
Bullera (15, 76)	5	15	*Rhodotorula* (32, 49)	18	31
Bulleromyces (1, 2)	1	2	*Sakaguchia* (1, 1)	0	0
Cryptococcus (37, 101)	28	91	*Sirobasidium* (1, 1)	1	1
Cystofilobasidium (4, 4)	2	2	*Sphacelotheca* (1, 1)	0	0
Erythrobasidium (1, 1)	0	0	*Sporidiobolus* (4, 8)	2	4
Fellomyces (4, 4)	0	0	*Sporobolomyces* (28, 50)	8	10
Fibulobasidium (1, 1)	1	1	*Sterigmatomyces* (2, 2)	0	0
Filobasidium (2, 2)	2	2	*Sterigmatosporidium* (1, 1)	1	1
Holtermannia (1, 1)	1	1	*Sympodiomycopsis* (1, 1)	0	0
Kockovaella (2, 2)	0	0	*Tilletiopsis* (7, 8)	3	3
Kondoa (1, 1)	0	0	*Trichosporon* (16, 31)	10	12
Kurtzmanomyces (1, 1)	1	1	*Trimorphomyces* (1, 1)	0	0
Leucosporidium (3, 3)	1	1	*Tsuchiyaea* (1, 1)	1	1
Mrakia (4, 4)	1	1	*Udeniomyces* (3, 15)	1	1
Pseudozyma (1, 1)	0	0	*Xanthophyllomyces* (1, 5)	1	1

The toxin activity was reduced at elevated temperatures, and at 100°C it was completely lost after 10 min of incubation. The killer factor of *B. alba* was resistant to proteases (pepsin, trypsin, α-chymotrypsin and pronase E (Sigma) except for proteinase K (Merck)). Inhibitory activity of toxin-containing filtrate was lost after addition of both living or dead (incubated at 100°C, 10 min) cells of sensitive yeast, but neither chitin (from crab shells), β-gluscan (from barley) nor mannan (from *Saccharomyces cerevisiae* Meyen ex Hansen) bound the toxin.

The killer phenotype of the *B. alba* JCM 7495 was cureless. One hundred and twenty clones were obtained after treatment of this strain with cycloheximide or at elevated temperature but all of them retained the killer activity.

The killer toxin secreted by *B. alba* JCM 7495 had a broad spectrum of action. It inhibited the growth of many species of both ascomycetous and basidiomycetous yeasts (Tables 1, 2). Of 251 species examined (475 strains belonging to 65 genera), 51% were sensitive to this toxin. The percentages of ascomycetous, tremellaceous, sporidiobolaceous and ustilaginaceous species sensitive to the *B. alba* toxin were 48, 59, 41 and 30, respectively. Tremellaceous yeasts were, as a rule, highly sensitive, while most species of different taxonomic affinity usually displayed a weak sensitivity. The exceptions to this, showing high sensitivity to the *B. alba* toxin, among ascomycetous organisms were species of *Lipomyces*, *Myxozyma*, *Zygozyma* and *Nadsonia*; among sporidiobolaceous yeasts, *Bensingtonia ciliata* Ingold, *Ben. ingoldii* Nakase et Itoh, *Ben. naganoensis* (Nakase et Suzuki) Nakase et Boekhout, *Leucosporidium antarcticum* Fell, Statzell, Hunter et Phaff, *Rhodotorula bogoriensis* (Deinema) von Arx et Weijman, *Rh. fragariae* (Barnett et Buhagiar) Rodrigues de Miranda et Weijman, *Rh. hylophila* (van der Walt, Scott et van der Klift) Rodrigues de Miranda et Weijman, *Rh. lignophila* (Dill et al.) Roeijmans et al., *Rh. sonckii* (Hopsu-Havu, Tunnela et Yarrow) Rodrigues de Miranda et Weijman, *Sporobolomyces foliicola* Shivas et Rodrigues de Miranda and *Sp. singularis* Phaff et do Carmo-Sousa; and among ustilaginaceous yeasts, *Rh. acheniorum* (Buhagiar et Barnett) Rodrigues de Miranda, *Rh. bacarum* (Buhagiar) Rodrigues de Miranda et Weijman, *Rh. hinnulea* (Shivas et Rodriues de Miranda) Rodrigues de Miranda et Weijman and *Rh. phylloplana* Shivas et Rodrigues de Miranda) Rodrigues de Miranda et Weijman.

This toxin was active against pathogenic yeast *Cryptococcus neoformans* (Sanfelice) Vuillemin. All 18 strains of *Cr. neoformans* (Sanfelice) Vuillemin var. *gattii* Vanbreuseghem et Takashio (serotypes B and C) were sensitive, but 10 of 47 strains of *Cr. neoformans* (Sanfelice) Vuillemin var. *neoformans* (serotypes A, D and AD) were found to be resistant (Table 3). Weak activity was usually observed against pathogenic species of *Trichosporon* (*Tr. asahii* Akagi, *Tr. asteroides* (Rischin) Ota, *Tr. cutaneum* (de Beurmann, Gougerot et Vaucher) Ota, *Tr. mucoides* Gueho et Smith, *Tr. ovoides* Behrend, *Candida albicans* (Robin) Berkhout and *C. krusei* (Castellani) Langeron et Guerra (*Issatchenkia orientalis* Kudriavzev). The species *C. glabrata* (Anderson) Meyer et Yarrow, *C. (Clavispora) lusitaniae* Rodrigues de Miranda and *C. tropicalis* (Castellani) Berkhoiut were insensitive.

Discussion

The broad action spectrum, increased thermostability, resistance to many proteases and relatively small molecular mass indicate that the killer toxin produced by *B. alba* is a microcin (Baquero and Moreno, 1984). In these

Table 2. Killing pattern of the *Bullera alba* microcin among ascomycetes.

Genera (number of species and strains examined)	Number of sensitive species	strains	Genera (number of species and strains examined)	Number of sensitive species	strains
Ambrosiozyma (1, 1)	0	0	*Mastigomyces* (1, 1)	0	0
Arthroascus (1, 1)	1	1	*Metschnikowia* (1, 1)	1	1
Arxulla (1, 1)	0	0	*Myxozyma* (3, 3)	3	3
Babjevia (1, 1)	1	1	*Nadsonia* (1, 1)	1	1
Botryoascus (1, 1)	0	0	*Pichia* (2, 2)	1	1
Candida (5, 5)	1	1	*Saccharomyces* (1, 1)	0	0
Citeromyces (1, 1)	0	0	*Saccharomycopsis* (2, 2)	0	0
Clavispora (1, 1)	0	0	*Saturnospora* (1, 1)	0	0
Debaryomyces (10, 30)	7	19	*Schizosaccharomyces* (1, 1)	0	0
Dipodascus (1, 1)	0	0	*Schwanniomyces* (1, 2)	0	0
Endomyces (1, 1)	0	0	*Stephanoascus* (2, 2)	1	1
Guilliermondella (1, 1)	0	0	*Wickerhamia* (1, 1)	1	1
Hanseniaspora (1, 1)	0	0	*Williopsis* (1, 1)	0	0
Issatchenkia (1, 1)	1	1	*Yarrowia* (1, 1)	0	0
Lipomyces (5, 5)	5	5	*Zygoascus* (1, 1)	0	0
Lodderomyces (1, 1)	1	1	*Zygosaccharomyces* (1, 1)	0	0
			Zygozyma (2, 2)	2	2

Table 3. Anti-*Cryptococcus neoformans* activity of the *Bullera alba* microcin.

Serotype	Origin	Number of strains examined	Number of strains		
			sensitive	weakly sensitive	resistant
		Cryptococcus neoformans var. *neoformans*			
A	Brazil	6	3	3	0
	Japan	5	0	2	3
	Thailand	6	1	5	0
	USA	4	2	1	1
D	Italy	13	3	6	4
	Japan	5	4	1	0
	USA	3	1	2	0
AD	Italy	3	0	1	2
	Japan	2	0	2	0
Total		47	14	23	10
		Cryptococcus neoformans var. *gattii*			
B	Australia	1	1	0	0
	USA	9	4	5	0
C	USA	8	5	3	0
Total		18	10	8	0

characteristics, the *B. alba* toxin is similar to the *Cr. humicola* (Daszewska) Golubev microcin, but the latter, which has a molecular mass of about 1 kDa, is more thermostable and has a broader action spectrum (Golubev and Shabalin, 1994). Like the *Cr. humicola* killers, in which no plasmids were detected, the microcin synthesis in *B. alba* appears also to be controlled by chromosomal genes.

The killing pattern of the toxin studied includes both ascomycetous and basidiomycetous yeasts though increased sensitivity was noted among tremellaceous organisms related taxonomically to *B. alba*. At the same time, among non-related yeasts, certain taxa display high sensitivity to the *B. alba* microcin. Among ascomycetous yeasts wide zones of growth inhibition were observed mainly in members of the family Lipomycetaceae (especially *Lipomyces japonicus* van der Walt, M. Th. Smith, Yamada et Nakase, *L. lipofer* Lodder et Kreger-van Rij ex Slooff and *L. starkeyi* Lodder et Kreger-van Rij) including the anamorphic genus *Myxozyma* (van der Walt, 1992) (Table 2).

In contrast to all other *Lipomyces* species, *L. anomalus* Babjeva et Gorin, which was reclassified recently into the new genus *Babjevia* (Smith et al., 1995), was very weakly sensitive to the *B. alba* microcin.

Almost all *Rhodotorula* spp. (Table 1) that were sensitive to this killer toxin (including weakly sensitive species *Rh. diffluens* (Ruinen) von Arx et Weijman, *Rh. phillyla* (van der Walt, Scott et van der Klift) Rodrigues de Miranda et Weijman and *Rh. pilatii* (Jacob et al.) Weijman) had been transferred from the genus *Candida* (Weijman et al., 1988). Unlike typical members of *Rhodotorula*, most of the sensitive species assimilate glucuronate (Golubev, 1989), often contain rhamnose in their exocellular polysaccharides (Golubev, 1995), and are located in different clusters on analysis of LSUrDNA sequences (Fell et al., 1995). These examples demonstrate that in some cases the differences in response to the *B. alba* microcin may be useful from a taxonomic viewpoint.

However, taking into account its broad action spectrum (Tables 1, 2), increased thermostability, resistance to many proteases and expression of activity within a rather wide range of pH, the toxin studied is evidently of more interest for yeast ecology. Some authors suggest that killer yeasts may affect the composition of yeast communities (Ganter and Starmer, 1992). At present, the absence of data on the proportion of *B. alba* killers in natural populations does not allow the role of microcin production in yeast communities to be estimated, although the world-wide distribution of this species and the higher frequency of killers in natural habitats than in culture collections (Young, 1987) give some basis for speculation. *Bullera alba* is the most common species of *Bullera* on both living and dead plants. Di Menna (1971) found the proportion of this species in the yeast population on leaves of white clover (New Zealand) to be 5% (more than 60,000 cells/g). *Bullera alba* was isolated from 28% of samples of dead rice leaves (Japan) and its isolates constituted 15% of all isolates of ballistosporeforming organisms and 27% of all isolates of *Bullera* spp. (Nakase and Suzuki, 1985).

The increased frequency of mycotic diseases in recent decades and the rather restricted number of antifungal drugs are among the reasons why special attention should be paid to the activity of the microcin found against pathogenic yeasts. The case of common life-threating fungal infection in AIDS patients, *Cr. neoformans* (Powderly, 1993) was the most susceptible to it. Of the strains tested, including clinical isolates, 83% showed sensitivity to the *B. alba* microcin (Table 3). The findings presented suggest that this microcin or its derivatives have potential as antifungal agents against

pathogenic yeasts.

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Literature cited

Baquero, F. and Moreno, F. 1984. The microcins. FEMS Microbiol. Lett. **23**: 117–124.

Di Menna, M. E. 1971. The mycoflora of leaves of pasture plants in New Zealand. In: Ecology of leaf surface microorganisms, (ed. Preece, T. F. and Dicknson, C. H.), pp. 159–174. Academic Press, London.

Fell, J. W., Boekhout, T. and Freshwater, D. W. 1995. The role of nucleotide analysis in the systematics of the yeast genera *Cryptococcus* and *Rhodotorula*. Stud. Mycol. **38**: 129–146.

Ganter, P. F. and Starmer, W. T. 1992. Killer factor as a mechanism of interference competition in yeasts associated with cacti. Ecology **73**: 54–67.

Golubev, W. I. 1989. Catabolism of *i*-inositol and taxonomic significance of the ability of yeasts to assimilate D-glucuronate. Mikrobiologiya **58**: 276–283.

Golubev, W. I. 1995. Basidiomycetous yeasts (Taxonomy and ecology). PhD thesis, Inst. Biochem. Physiol. Microorganisms, Pushchino, Russia.

Golubev, W. I. and Boekhout, T. 1995. Sensitivity to killer toxins as a taxonomic tool among heterobasidiomycetous yeasts. Stud. Mycol. **38**: 47–58.

Golubev, W. I. and Shabalin, Y. 1994. Microcin production by the yeasts *Cryptococcus humicola*. FEMS Microbiol. Lett. **119**: 105–110.

Koltin, Y. 1988. The killer system of *Ustilago maydis*: Secreted polypeptides encoded by viruses. In: Viruses of fungi and simple eukaryotes, (ed. by Koltin, Y. and Leibowitz, M. J.), pp. 209–242. Marcel Dekker, New York.

Konisky, J. 1982. Colicins and other bacteriocins with established modes of action. Annu. Rev. Microbiol. **36**: 125–144.

Makower, M. and Bevan, E. A. 1963. The physiological basis of the killer character in yeast. Proc. XIth Inter. Congr. Genetics **1**: 202–203.

Mizutani, A., Hagiwara, H. and Yanagisawa, K. 1990. A killer factor produced by the cellular slime *Polyspondylium pallidum*. Arch. Microbiol. **153**: 413–416.

Nakase, T. and Suzuki, M. 1985. Ballistospore-forming yeasts found on the surface of the Japanese rice plant, *Oryza sativa* L. J. Gen. Appl. Microbiol. **31**: 457–474.

Powderly, W. G. 1993. Cryptococcal meningitis and AIDS. Clin. Infect. Dis. **17**: 837–842.

Quackenbush, R. L. 1988. Endosymbionts of killer paramecia. In: Paramecium, (ed. by Gottz, H. D.), pp. 406–418. Springer, Berlin.

Smith, M. Th., van der Walt, J. P. and Batenburg-van der Vegte, W. H. 1995. *Babjevia* gen. nov. – a new genus of the Lipomycetaceae. Antonie van Leeuwenhoek **67**: 177–179.

Walt, J. P. van der 1992. The Lipomycetaceae, a model family for phylogenetic studies. Antonie van Leeuwenhoek **62**: 247–250.

Weijman, A. C. M., Rodrigues de Miranda, L. and Walt, J. P. van der 1988. Redefinition of *Candida* Berkhout and the consequent emendation of *Cryptococcus* Kützing and *Rhodotorula* Harrison. Antonie van Leeuwenhoek **54**: 545–553.

Young, T. W. 1987. Killer yeasts. In: The yeasts, vol. 2, (ed. by Rose, A. H. and Harrison, J. S.), pp. 131–163. Academic Press, London.