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EXPERIMENTAL ARTICLES

Anti-Tremellomycetes Activity of *Cryptococcus pinus* Mycocin

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Abstract—The mycocin with a molecular mass of at least 15 kDa secreted by *Cryptococcus pinus* exhibited fungicidal activity at pH values below 6.5. It was thermolabile and resistant to proteases. In the class Tremellomycetes the species of the orders *Filobasidiales* and *Tremellales* are sensitive to this mycocin.

Key words: mycocinotyping, taxonomy, identification, *Filobasidiales, Tremellales.*

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Antagonistic relationships caused by the formation of antibiotic substances are quite common among microorganisms, including yeasts [1]. The latter, in particular, often exhibit mycocinogeny, i.e., synthesis of proteinaceous substances termed mycocins (killer toxins) [2]. Their fundamental peculiarity is taxon specificity: only the organisms phylogenetically related to the mycocins producers are sensitive to these compounds. Mycocinogeny obviously should enhance the producer's competitiveness in microbial communities against related species with similar requirements and ecological niches. The wide occurrence of mycocinogenic yeasts revealed by now [3, 4] prompts reexamination of the established ideas of the role of these unicellular fungi in natural biocenoses. Moreover, the taxon specificity of mycocins can be used for taxonomic and diagnostic purposes [5, 6]. The latter is quite relevant, because the gap between microbial taxonomy and identification (its practical application) is presently increasing. Quite often, the newly described taxa revealed by molecular biological methods are almost indistinguishable by generally accepted standard methods. The high cost and laborious of molecular biological techniques limits their use for research (e.g., ecological, epidemiological) dealing with hundreds or thousands of isolates. At present, diagnostic criteria are urgently needed, which would correlate with the modern taxonomic data and, at the same time, provide the possibility of simultaneous testing of a great number of cultures in minimally equipped laboratories. In the case of yeast fungi, mycocinotyping (determination of sensitivity to mycocins) is one of these approaches.

The degree of relationship of sensitive organisms may be quite different depending on the mycocin, from strains within a species to representatives of related orders. Thus, the application of mycocins for diagnostic purposes requires preliminary determination of the taxonomic level where they exhibit their activity. Besides, it is very important to select the appropriate mycocinogenic cultures: they must be different but, to some extent, taxonomically related to the studied strains. For example, it makes no sense to use basidiomycetous yeasts for testing ascomycetous ones and vice versa. At the same time, the resolution of mycocinotyping increases when the number of mycocins is greater and their set is relevant [7]. A substantial number of mycocinogenic strains with known and various action spectra are therefore required for identification.

Mycocinogeny was revealed in the recently described species *Cryptococcus pinus* Golubev and Pfeiffer [8]. The present work pursued the study of some characteristics of the secreted mycocin and detailed examination of the action spectrum as compared with the mycocins of other *Cryptococcus* species [7, 9].

MATERIALS AND METHODS

Strains. The cultures used in this work were obtained mainly from the All-Russian Collection of Microorganisms (VKM, http:/www.vkm.ru).

Obtaining and characterization of the toxin. Strain VKM Y-2958 was incubated for a week on a shaker (150 rpm, 20°C) in glucose–peptone medium (pH 4.5) [9]. The cells were separated by centrifugation (5000 *g*, 10 min), the supernatant was filtered through GF/A glass-fiber filter paper (Whatman, United Kingdom), and the resultant active culture liquid was used to assess the action of the toxin on the viability of sensitive yeasts determined by plating on malt agar (MA) in the course of incubation. This culture liquid was also used to test the toxin's resistance to elevated temperature and proteolysis by the agar well method. For estimation of the molecular mass of the toxin, strain VKM Y-2958 was grown for a week on glucose–peptone agar

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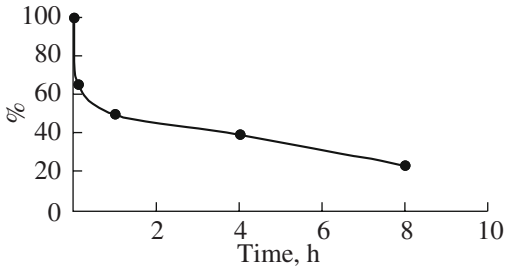

Figure. The dynamics of cell death of *Cryptococcus terreus* VKM Y-2253 (% of the initial concentration (4×10^3 cells/ml)) at incubation (20°C) in a mycocin-containing culture liquid of *Cryptococcus pinus* VKM Y-2958.

(GPA, pH 4.5), covered with dialysis membranes that were permeable for substances of a known molecular mass (Spectrum, United States), then the membranes were removed together with the streak culture of this strain, and the GPA surface was inoculated with a lawn of sensitive yeasts.

Table 1. Ascomycetous yeasts (54 species) tested for the sensitivity to the *Cryptococcus pinus* mycocin

Aciculoconidium	*Kluyveromyces*	*Schizoblastosporion*
Arthroascus	*Lachancea*	*Schizosaccharomyces*
Arxula	*Lipomyces*	*Stephanoascus*
Ambrosiozyma	*Lodderomyces*	*Taphrina*
Arxiozyma	*Mastigomyces*	*Tetrapisispora*
Brettanomyces	*Metschnikowia*	*Torulaspora*
Candida	*Nadsonia*	*Trigonopsis*
Citeromyces	*Nakaseomyces*	*Vanderwaltozyma*
Clavispora	*Naumovia*	*Wickerhamia*
Debaryomyces	*Nematospora*	*Wickerhamiella*
Dekkera	*Oosporidium*	*Williopsis*
Endomyces	*Pachysolen*	*Wingea*
Guilliermondella	*Pichia*	*Yarrowia*
Hanseniaspora	*Protomyces*	*Zygoascus*
Hormoascus	*Saccharomyces*	*Zygosaccharomyces*
Issatchenkia	*Saccharomycodes*	*Zygotorulaspora*
Kazachastania	*Saturnispora*	*Zygowilliopsis*
Kloeckera		

Elimination of antifungal activity. The cell suspension of strain VKM Y-2958, 0.1 ml (10^4 cells/ml), was plated on MA and incubated at the maximal growth temperature. The grown colonies selected at random were tested for the presence of antifungal activity.

The sensitivity of 3 day cultures grown on MA was tested by the "culture-to-culture" method on GPA with citrate–phosphate buffer (pH 4.5) [9].

RESULTS

Among 15 *C. pinus* isolates [8], antifungal activity was detected only for the strain Ps-38 (=VKM Y-2958). This activity was observed only at acidic pH values of the medium, within the range of 3.5 to 6.5. Judging by the width of the growth inhibition zones, it is maximal at pH 4.5.

The secreted antifungal factor was resistant to the action of proteases E, P, XII, and XIX (Sigma, United States) but was completely inactivated after 5 min at 100°C. It did not diffuse through the dialysis membranes that retained the compounds with the molecular mass of 15 kDa.

This factor is lethal for sensitive yeasts, as can be seen from the blue edge on their lawn around the growth inhibition zone on GPA with methylene blue (0.03 g/l). Its direct fungicidal action is demonstrated in the figure.

The testing of more than 80 colonies grown at the maximal growth temperature for strain VKM Y-2958 (32°C) showed no loss of antibiotic activity.

The sensitivity to the studied factor was tested in 314 fungal strains belonging to 204 species of 120 genera. All representatives of *Ascomycota* (Table 1) were resistant to it; among *Basidiomycota*, the teleomorphic and anamorphic sensitive organisms belonged to the orders *Filobasidiales* and *Tremellales* of the class *Tremellomycetes* (Tables 2, 3). The members of other classes and orders of basidiomycetes were resistant or sometimes weakly sensitive. Within the polyphyletic genus *Cryptococcus* [10], the situation is similar; almost all filobasidaceous and tremellaceous species were sensitive, while the species phylogenetically belonging to the orders *Cystofilobasidiales* and *Trichosporonales* were resistant or sometimes weakly sensitive (Table 4).

DISCUSSION

At present, yeast fungi are known to produce two types of extracellular antifungal compounds: mycocins and glycolipids [1]. Although both of them are active at acidic pH values of the medium, they differ in molecular mass, sensivity to elevated temperatures and proteases, and in the ranges of action spectra. Thermolability, considerable molecular mass (at least 15 kDa) and, above all, the taxonomic specificity of the action spectrum make it possible to classify the antifungal factor

Table 2. The action spectrum of the *Cryptococcus pinus* mycocin among basidiomycetes

	Agaricomycotina			*Kondoa malvinella* VKM Y-1568, 1569	–
	Agaricomycetes			Atractiellomycetes	
Sebacinales	*Sebacina penetrans* VKM Y-2689	–	Atractiellales	*Atractogloea stillata* VKM Y-2693	w
	Dacrymycetes			Cystobasidiomycetes	
Dacrymycetales	*Dacrymyces stillatus* VKM F-2953	–	Erythrobasidiales	*Erythrobasidium hasegawianum* VKM Y-2802	w
	Tremellomycetes			*Sakaguchia dacryoides* VKM Y-2702, 2703	w
Cystofilobasidiales	*Cystofllobasidium bisporidii* VKM Y-2700	–		Microbotryomycetes	
	Mrakia curviuscula VKM Y-2953	–	Leucosporidiales	*Curvibasidium pallidicorallinum* VKM Y-2284, 2861	–
	M. frigida VKM Y-1455	–		*Leucosporidium scottii* VKM Y-68, 2774	–
	M. gelida VKM Y-2699	–		*Mastigobasidium intermedium* VKM Y-2720	–
	Xanthophyllomyces dendrorhous VKM Y-2274, 2786	–	Microbotryales	*Microbotryum scorzonerae* RBF 855	–
Filobasidiales	*Filobasidium capsuligenum* VKM Y-1439	–		*M. silene-inflatae* VKM F-2974, 3319	–
	F. capsuligenum VKM Y-1513	+		*M. vinosa* VKM F-2973	w
	F. elegans VKM Y-2916	–		*M. violaceum* VKM F-2976, 3318	–
	F. floriforme VKM Y-2257	+		*Sphacelotheca polygoni-persicariae* VKM Y-2691	w
	F. globisporum VKM Y-2798	w	Sporidiobolales	*Rhodosporidium babjevae* VKM Y-2275, 2276	–
	F. uniguttulatum VKM Y-1597	+		*R. toruloides* VKM Y-333, 334	–
Tremellales	*Bulleromyces albus* VKM Y-2141	+		*Sporidiobolus johnsonii* VKM Y-2606	–
	Fibulobasidium inconspicuum VKM Y-2732	+		*S. salmonicolor* VKM Y-679, 685	–
	Filobasidiella neoformans IGC 3957, 4208	+		Pucciniomycetes	
	Holtermannia corniformis VKM Y-2803, 2804	–	Septobasidiales	*Septobasidium carestianum* VKM Y-2690	w
	Kwoniella mangroviensis VKM Y-2959, 2960	–	Platygloales	*Platygloea peniophorae* VKM Y-2688	–
	Papiliotrema bandonii VKM Y-2917	+	Puccinales	*Endophyllum sempervivi* VKM Y-2695	w
	Sirobasidium magnum VKM Y-2730, 2731	w		*Gymnosporangium clavarieforme* VKM Y-2687	w
	Sterigmatosporidium polymorphum VKM Y-2585	+		*Puccinia bupleuri* VKM F-2979	w
	Tremella aurantia VKM Y-2678	+		*P. suaveolens* VKM F-2980	w
	T. encephala VKM Y-2679	+		***Ustilaginomycotina***	
	T. foliacea VKM Y-2680	+		Exobasidiomycetes	
	T. fuciformis VKM Y-2761	+	Entylomatales	*Entyloma gaillardianum* RBF 833	–
	T. indecorata VKM Y-2775	+	Exobasidiales	*Exobasidium vaccinii* VKM F-2957	–
	T. mesenterica VKM Y-2681	+	Microstromatales	*Microstroma juglandis* VKM Y-2696	+
	T. mycophaga VKM Y-2682	+	Tilletiales	*Neovossia setariae* GD 1751	–
	T. samoensis VKM Y-2773	+		*Tilletia caries* VKM F-2964	–
	T. simplex VKM Y-2683	+		Ustilaginomycetes	
	T. subanomala VKM Y-2797	+	Ustilaginales	*Farysia thuemenii* VKM Y-2686	w
	Trimorphomyces syzygius VKM Y-2697	+		*Sporisorium transfissum* VKM Y-2692	–
	Pucciniomycotina			*Ustilago maydis* VKM F-2971	–
	Agaricostilbomycetes				
Agaricostilbales	*Agaricostilbum pulcherrimum* VKM Y-2684	–			

Note: +, sensitive; w, weakly sensitive; –, insensitive.

Table 3. The action spectrum of *C. pinus* mycocin among anamorphic basidiomycetes

	Agaricomycotina	
	Tremellomycetes	
Cystofilobasidiales	*Itersonilia perplexans* VKM Y-2734-2736	w
	Tausonia pamirica VKM Y-2836	w
	Trichosporon pullulans VKM Y-2303, 2833	–
	Udeniomyces megalosporus VKM Y-2718	+
	Ud. puniceus VKM Y-2605	–
	Ud. pyricola VKM Y-2069, 2662, 2852	–
Tremellales	*Bullera dendrophila* VKM Y-2167	–
	B. hannae VKM Y-2832	+
	B. huianensis VKM Y-2831	+
	B. mrakii VKM Y-2827	+
	B. miyagiana VKM Y-2769	+
	B. oryzae VKM Y-2704	+
	B. pseudoalba VKM Y-2719	–
	B. sinensis VKM Y-2717	–
	B. sinensis var. *lacti*s VKM Y-2826	+
	B. taiwanensis VKM Y-2945, 2952	+
	B. unica VKM Y-2830	+
	B. variabilis VKM Y-2815	+
	Dioscegia crocea VKM Y-2801, 2825	–
	D. hungarica VKM Y-1600, 2741, 2742, 2800	+
	Fellomyces polyborus VKM Y-2169	+
	Kockovaella thailandica VKM Y-2805	+
	Tsuchiyaea wingfieldii VKM Y-2816	+
Trichosporonales	*Trichosporon cutaneum* VKM Y-181, 809	–
	Pucciniomycotina	
	Agaricostilbomycetes	
Agaricostilbales	*Bensingtonia ingoldii* VKM Y-2765	–
	Kurtzmanomyces nectairii VKM Y-2214	–
	Sterigmatomyces halophilus VKM Y-2666	–
	Microbotryomycetes	
Leucosporidiales	*Sporobolomyces singularis* VKM Y-1299	–
	S. tsugae VKM Y-1280	–
Sporidiobolales	*Rhodotorula glutinis* VKM Y-262, 332	–
	Ustilaginomycotina	
	Exobasidiomycetes	
Exobasidiales	*Tilletiopsis albescens* VKM Y-2822	w
Georgefisheriales	*Tilletiopsis flava* VKM Y-2823	–
Microstromatales	*Sympodiomycopsis paphiopedili* VKM Y-2817	–
	Ustilaginomycetes	
Ustilaginales	*Pseudozyma graminicola* VKM Y-2938	–
	P. prolifica VKM Y-2835	–

Table 4. The intrageneric action spectrum of *C. pinus* mycocin

Cystofilobasidiales		Tremellales	
C. aquaticus VKM Y-2428, 2710	–	*C. allantoinivorans* VKM Y-2933	+
C. huempii VKM Y-2637	w	*C. amylolentus* VKM Y-2227	–
C. macerans VKM Y-1263, 1328, 1989, 2298, 2740, 2929	–	*C. aureus* VKM Y-328	+
Filobasidiales		*C. carnescens* VKM Y-720	+
Cryptococcus aerius VKM Y-1540, 1994	+	*C. dimennae* VKM Y-1644	+
C. albidus VKM Y-2223	+	*C. festucosus* VKM Y-2930	+
C. albidus var. *kuetzingii* VKM Y-1646	+	*C. flavescens* VKM Y-1032, 1595, 2240, 2241	+
C. albidus var. *ovalis* VKM Y-1539, Pa-19	+	*C. flavus* VKM Y-2232	w
C. arrabidensis VKM Y-2894	–	*C. foliicola* VKM Y-2947	+
C. bhutanensis VKM Y-1643	+	*C. heveanensis* VKM Y-1457, 1991, 1992, 2239,2245, 2255	–
C. chernovii VKM Y-2890	–	*C. laurentii* VKM Y-1627, 1628, 1656, 1665	+
C. cylindricus VKM Y-2895	+	*C. luteolus* VKM Y-1596, 2032	–
C. diffluens VKM Y-1592, 1966	w	*C. marinus* VKM Y-1587	–
C. filicatus VKMY-2954	+	*C. mycelialis* VKM Y-2863	+
C. filicatus var. *pelliculosus* VKM Y-2955, At-2, 6	–	*C. nemorosus* VKM Y-2906	+
C. fuscescens VKM Y-2600	+	*C. nyarrowii* VKM Y-2901	+
C. gastricus VKM Y-1030, 2000, 2256, 2934	+	*C. paraflavus* VKM Y-2923	+
C. gilvescens VKM Y-2748	+	*C. peneaus* VKMY-1291	+
C. liquifaciens VKM Y-744	+	*C. perniciosus* VKM Y-2905	+
C. magnus VKM Y-1534, 1550, 2243, 2246	+	*C. pinus* Ps-35, 40A, 51, 52, 55, 63	–
C. oeirensis VKM Y-2893	+	*C. podzolicus* VKM Y-2248	+
C. phenolicus VKM Y-2892	+	*C. skinneri* VKM Y-1283	+
C. saitoi VKM Y-2896	w	*C. victoriae* VKM Y-2936	+
C. silvicola VKM Y-2939	+	*C. watticus* VKM Y-2937	+
C. stepposus VKM Y-2918, PTZ-289	–	Trichosporonales	
C. terreus VKM Y-2253	+	*C. curvatus* VKM Y-2230	–
C. terricola VKM Y-1598	+	VKM Y-2228, 2229, 2236	w
C. uzbekistanensis VKM Y-2891	+	*C. haglerorum* VKM Y-2932	–
C. vishniacii VKM Y-2663, 2799	w	*C. humicola* VKM Y-926, 1613, 2238	w
C. wieringae VKM Y-2915	+		

secreted by *C. pinus* VKM Y-2958 with mycocins. The latter are usually liable to proteolysis; however, some of the described mycocins are resistant to proteases due to a highly compact structure [11]. From the practical point of view, protease resistance obviously enhances the attractiveness of *C. pinus* mycocin as an antifungal agent, in particular, against the cryptococcosis pathogen, *C. neoformans* (teleomorph *Filobasidiella neoformans*), which is sensitive to it (Table 2).

The synthesis of mycocin by *C. pinus* is probably determined by chromosomal genes, because the incubation at elevated temperatures, which efficiently induces elimination of the cytoplasmic genetic elements, did not lead to growth of the cultures devoid of the antifungal activity.

The rather broad action spectrum of *C. pinus* mycocin includes representatives of 18 genera of tremellomycetes (Tables 2–4), and the sensitivity to it may be considered as a specific feature of the orders *Filobasidiales* and *Tremellales*. As a taxonomic tool, it is probably most useful for assiqument to these orders of anamorphic yeasts, usually dominating in the epiphyte and soil communities [4, 10]. Among the currently accepted orders of *Tremellomycetes*, the representatives of *Cystofilobasidiales* and *Trichosporonales* are resistant or weakly sensitive to this mycocin. The differences between the *Cryptococcus* species in their resistance to *C. pinus* mycocin are additional evidence of the polyphyletic nature of this genus (Table 4).

Among the *Cryptococcus* mycocins with the action spectra relatively completely characterized (*C. aquaticus* (Jones et Slooff) Rodrigues de Miranda et Weijman, *C. laurentii* (Kufferath) Skinner, *C. nemorosus* Golubev et al., *C. perniciosus* Golubev et al., and *C. podzolicus* (Bab'eva et Reshetova) Golubev), the mycocins produced by *C. laurentii* strains VKM Y-1627 and VKM Y-1665 are the closest in this respect to the one under study [9]. However, unlike them, among the *Tremellomycetes C. pinus* mycocin is active against *C. carnescens* (Verona and Luchetti) Takashima et al., *C. nyarrowii* Thomas-Hall et Watson, *C. victoriae* Montes et al., *Fellomyces polyborus* (Scott et van der Walt) Yamada and Banno, *Tremella mesenterica* Retzius : Fries, but inactive against *Bullera pseudoalba* Nakase et Suzuki, *C. amylolentus* (van der Walt et al.) Golubev, *C. arrabidensis* Fonseca et al., *C. chernovii* Fonseca et al., *C. heveanensis* (Gronewege) Baptist et Kurtzman, *C. luteolus* ((Saito) Skinner), *Kwoniella mangroviensis* Statzell-Talman et al., and *Trichosporon pullulans* (Lindner) Diddens et Lodder. These differences may be used for diagnostic purposes. They allow, for example, differentiation between the phenotypically very similar species of the *C. laurentii* complex [12]: *C. carnescens, C. victoriae*, and *C. heveanensis*.

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REFERENCES

1. Golubev, W.I., Antagonistic Interactions among Yeasts, in *Biodiversity and Ecophysiology of Yeasts*, Rosa, C.A. and Peter, G., Eds., Berlin: Springer, 2006, pp. 197–219.
2. Golubev, W.I, Mycocins (Killer Toxins), in *The Yeasts, a Taxonomic Study*, Kurtzman, C.P. and Fell, J.W., Eds., Amsterdam: Elsevier, 1998, pp. 55–62.
3. Golubev, W.I. and Churkina, L.G., Wide Distibution of Killer Strains in the Yeast *Rhodotorula mucilaginosa* (Jorgensen) Harrison, *Izv. AN SSSR, Ser. Biol*., no. 6, pp. 854–861.
4. Golubev, W.I. and Golubeva, E.V., Yeast Fungi of the Steppe and Forest Phytocenoses of the Prioksko-Terrasny Biosphere Reserve, *Mikol. Fitopatol.*, 2004, vol. 38, no. 6, pp. 20–27.
5. Golubev, W.I and Boekhout, T., Sensitivity to Killer Toxins as a Taxonomic Tool Among Heterobasidiomycetous Yeasts, *Studies in Mycology*, 1995, no. 38, pp. 47–58.
6. Buzzini, P., Turchetti, B., and Vaughan-Martini, A.E., The Use of Killer Sensitivity Patterns for Biotyping Yeast Strains: the State of the Art, Potentialities and Limitation, *FEMS Yeast Res.*, 2007, vol. 7, no. 6, pp. 749–760.
7. Golubev, W.I. and Golubeva, E.V., Mycocinotyping of Filobasidiaceous Species of the Genus *Cryptococcus* Vuillemin, *Mikol. Fitopatol.*, 2006, vol. 40, no. 6, pp. 480–486.
8. Golubev, W.I., Pfeiffer, I., and Tomashevskaya, M.A., *Cryptococcus pinus* sp. nov., an Anamorphic Basidiomycetous Yeast Isolated from Pine Litter, *Int. J. Syst. Evol. Microbiol.*, 2008, vol. 58, no. 8, pp. 1968–1971.
9. Golubev, W.I. and Kuznetsova, L.B., Formation and Action Spectrum of Mycocins from Basidiomycete Yeast *Cryptococcus laurentii* (Kufferath) Skinner, *Mikrobiologiya*, 1989, vol. 58, no. 6, pp. 980–984.
10. Golubev, W.I., Genus *Cryptococcus* Vuillemin and Its Type Species *Cryptococcus neoformans* (Sanfelice) Vuillermin, in *Novoe v sistematike i nomenklature gribov* (Advances in the Systematics and Nomenclature of Fungi), D'yakov, Yu.T. and Sergeev, Yu.V., Eds., Moscow: Natl. Acad. Mycol., 2003, pp. 357–381.
11. Gu, F., Khimani, A., Rone, S.G., Flurkey, W.H., Bozarth, R.F., and Smith, T.J., Structure and Function of a Virally Encoded Fungal Toxin from *Ustilago Maydis*: a Fungal and Mammalian Ca^{2+} Channel Inhibitor, *Structure*, 1995, vol. 3, no. 8, pp. 805–814.
12. Takashima, M., Sugita, T., Shinoda, T., and Nakase, T., Three New Combinations from the *Cryptococcus laurentii* Complex: *Cryptococcus aureus, Cryptococcus carnescens*, and *Cryptococcus peneaus, Int. J. Syst. Evol. Microbiol.*, 2003, vol. 53, no. 4, pp. 1187–1194.