Investigation of a killer strain of *Zygosaccharomyces bailii*

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The yeast *Zygosaccharomyces bailii* strain 412 was found to liberate a killer toxin (KT412) lethal to sensitive strains of *Saccharomyces cerevisiae* and *Candida glabrata*. Culture supernatants of the killer strain were concentrated by ultrafiltration and the extracellular protein was purified by gel filtration and ion-exchange chromatography. Gel filtration and SDS-PAGE of the electrophoretically homogeneous killer protein indicated an apparent molecular mass of 10 kDa. The killer toxin KT412 is probably not glycosylated since it did not show any detectable carbohydrate structures. KT412 was bound to sensitive but not to resistant yeast cells. The mannan, and not the glucan, fraction of the cell wall of the sensitive yeast was the primary target for the killer toxin binding. The killer strain *Z. bailii* 412 contained three double-stranded RNA plasmids of 1.9, 2.9 and 4.0 kb. Curing by cycloheximide resulted in the concomitant loss of killer activity and the 1.9 kb dsRNA species that is therefore regarded as equivalent to the killer-toxin-coding M-plasmids of *S. cerevisiae*.

Introduction

The phenomenon of killer activity is frequent among yeasts. Occasionally almost half of new yeast isolates are killer strains (Radler *et al.*, 1985), and we have observed that many yeast strains with known genetic markers are killer strains. Killer strains produce protein or glycoprotein killer toxins that kill sensitive yeast strains. This phenomenon has been thoroughly reviewed (Bussey, 1981; Tipper & Bostian, 1984; Wickner, 1986; Young, 1987). Bevan & Makower (1963) first described killer strains of *Saccharomyces cerevisiae*. Thereafter, killer yeasts were observed in various yeast genera (Young & Philliskirk, 1977; Young & Yagiu, 1978; Middelbeek *et al.*, 1980; Radler *et al.*, 1985).

Different types of killer-toxin-producing yeast strains have been described within the genus *Saccharomyces*. In this genus, expression of the killer phenotype depends on the presence of two double-stranded RNA plasmids (called L- and M-plasmids). In *Kluyveromyces lactis*, two DNA plasmids determine the killer activity. Since dsRNA or dsDNA plasmids were only observed in some strains of the genera *Saccharomyces*, *Kluyveromyces* and

Pichia, it was assumed that the killer toxins of other genera are encoded by chromosomal genes (Young, 1987). This is not so, for it was later found that the killer phenotype of *Hanseniaspora uvarum* depended on dsRNA plasmids as well (Zorg *et al.*, 1988).

When screening yeast strains of the genus *Zygosaccharomyces* for killer activity, one strain (*Zygosaccharomyces bailii* 412) was found that showed a wide range of activity and contained dsRNA plasmids. As the killing spectrum of this strain was different from all killer toxins known so far, it was of interest to investigate this killer yeast by characterizing its killer toxin and determining the possible role of its RNA plasmids.

Methods

Micro-organisms, culture media, and determination of killer activity. The yeast strains used were from the collection of this institute. *Zygosaccharomyces bailii* 412 was originally obtained from Dr F. E. M. J. Sand, Bussum, the Netherlands; the various killer yeasts of the K-series and the sensitive strain *Saccharomyces cerevisiae* 452 (= NCYC 1006) were from Dr T. W. Young, Birmingham, UK.

Media, methods of cultivation and determination of killer activity have been described previously (Pfeiffer & Radler, 1984; Radler & Schmitt, 1987). YEP agar contained glucose (2%, w/v), peptone (2%, w/v), yeast extract (1%, w/v), agar (1.2%, w/v), and the pH was adjusted with potassium dihydrogen phosphate. YEPC medium was YEP with sodium citrate (1.92%, w/v) but without agar. The killer toxin is expressed in arbitrary units (aU). Based on the well test method (agar diffusion test), 10 aU correspond to the amount of killer toxin that causes an inhibition zone of 10 mm (20 mm measured diameter of

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Abbreviations: aU, arbitrary unit; ds, double-stranded; PAS, periodic acid/Schiff.

inhibition zone minus 10 mm diameter of the well) in the lawn of the sensitive strain *S. cerevisiae* 452.

Production and purification of the killer toxin (KT412) of *Z. bailii*. *Z. bailii* 412 was cultivated in 2 × 5000 ml synthetic B-medium (Pfeiffer & Radler, 1982) that was adjusted to pH 4.0, with the addition of 15% (v/v) glycerol, at 20 °C and with gentle stirring, for 4 d. After removal of the cells by centrifugation the culture liquid was concentrated by ultrafiltration, first with a Sartorius membrane to 400 ml, then with an Omega membrane (Filtron) to about 20 ml. The cut-off of both membranes was 10 kDa. After centrifugation, the concentrate was dialysed in Visking tubing type 36/32, 27 mm diam. (Roth), against 2 × 2000 ml 20 mM-malonate/phosphate buffer, pH 2.7, with the addition of 15% (v/v) glycerol and 4 M-urea, for 2 × 12 h. Before storage, the dialysate was filtered through a Millex-GV-filter (Millipore, 0.22 µm). Dialysate (5 ml) containing killer toxin was applied to a column (1.2 × 5 cm) of S-Sepharose, from which the killer toxin was eluted with a linear gradient of 0–1 M-NaCl (180 ml) in 20 mM-malonate/phosphate buffer [pH 2.7; 15% (v/v) glycerol; 4 M-urea]. Fractions of 6 ml were collected and the killer-toxin-containing fractions (nos 15 to 19) were pooled and dialysed overnight against 4 litres 10 mM-citrate/phosphate buffer [pH 3.7; 15% (v/v) glycerol] to remove the urea before lyophilization. The residue obtained after lyophilization was dissolved in a small volume of the same buffer. A sample (200 µl) of this preparation was applied to a column of Superose 12 (FPLC) from which the killer toxin was eluted with 100 mM-citrate/phosphate buffer (pH 3.7; 150 mM-NaCl; 4 M-urea). The fraction size was 1 ml and KT412 was found in fraction nos 15–17, of which fraction no. 16 was collected for further use. SDS-PAGE was performed according to Laemmli (1970), with minigels (Biometra) that were stained with the Silverstain kit for protein or with the periodic acid/Schiff (PAS) reagent according to Zacharius *et al.* (1969). The killer toxin K₂₈ from *S. cerevisiae* was isolated as described previously (Schmitt & Radler, 1987).

Isolation and detection of dsRNA plasmids. Cells of *Z. bailii* were extracted with phenol/chloroform, single-stranded RNA was removed by LiCl precipitation and the remaining nucleic acids were precipitated with ethanol as described by Schmitt & Tipper (1990). For electrophoresis 1% (w/v) agarose in TBE buffer (0.089 M-Tris/boric acid, pH 8.3, 0.0025 M-Na₂EDTA) was used as described by Maniatis *et al.* (1982).

Adsorption of killer toxin to cells, cell walls and cell wall polysaccharides. The experiments with the killer toxin of *Z. bailii* were performed as described by Schmitt & Radler (1987, 1988) for *S. cerevisiae*. Cell wall preparations of strains of *S. cerevisiae* were prepared as described previously (Radler & Schmitt, 1987). For the preparation of mannan, the yeast cells were extracted with NaOH (3%, w/v, at 75 °C for 2 h), and the mannan-copper complex was precipitated by adding Fehling's solution. The mannan was dissolved in 3 M-HCl and then precipitated with methanol/acetic acid (8:1, v/v). The glucan fractions were extracted from the alkali-insoluble residue with 0.5 M-acetic acid at 90 °C.

Results

Killer activity of *Zygosaccharomyces bailii*

When 40 strains of the genus *Zygosaccharomyces* were screened for killer activity using *Saccharomyces cerevisiae* 467 as the sensitive tester strain only strain 412 showed a distinct killer activity determined by the usual agar diffusion test. In cross-reactions with 21 killer yeasts of eight different genera and 20 non-killer strains of the

genera *Saccharomyces*, *Candida*, *Kluyveromyces* and *Saccharomycodes*, the killer yeasts *S. cerevisiae* 466 (K1), *Candida glabrata* 453 (K4), *Kluyveromyces marxianus* (K6) and *Hanseniaspora uvarum* 470, and the non-killers *Candida krusei* 15, and 6 out of 10 tested *S. cerevisiae* strains, were sensitive to the killer toxin of *Z. bailii*. The new killer strain of *Z. bailii* 412 was inhibited only by a newly isolated killer strain of *Torulaspora delbrueckii* 3/40. This pattern of cross-reactivity was different from that of all other available killer yeasts. Therefore, the killer activity of *Z. bailii* 412 was investigated further, as (to our knowledge) no killer strains of this genus have hitherto been described.

Characteristics of the killer toxin (KT412) of *Z. bailii* 412

We first demonstrated that the killer activity of *Z. bailii* 412 is caused by a protein (killer toxin), which will be designated KT412. The killer activity was destroyed (76–97%) by incubation with proteinase K and protease type XIII (Sigma). Other proteinases, such as pronase E and trypsin, had little effect (less than 15% inactivation after 24–48 h) on the activity of the native killer toxin. Yeast killer toxins are usually temperature- and pH-sensitive. The killer toxin of *Z. bailii* 412 is no exception. When kept at 37 °C for 3 h at pH 3.7, more than half of the activity was lost; at 50 °C about 90% of the activity was lost within 1 h. The killer toxin was most stable at pH 3 when kept at 4 °C for 24 h. Less than half of the activity was lost at pH 2 or pH 6; above pH 6 the inactivation increased (80% loss) significantly, and at pH 9 all activity was destroyed completely.

The killer toxin was precipitated from the culture fluid with 50% (v/v) ethanol. The activity could be concentrated by lyophilization, but ultrafiltration with a 10 kDa cut-off membrane was the preferred method. It was shown by isoelectric focusing that the isoelectric point of the killer toxin of *Z. bailii* was approximately pH 4.1. KT412 was found in the culture fluid. Cell extracts of washed cells of *Z. bailii* 412 contained only about 0.2% of the killer activity of the culture fluid.

Purification of the killer toxin KT412

The synthetic B-medium was used to facilitate the isolation of killer toxin. This avoided high molecular mass compounds in the culture medium. Glycerol was found to stabilize KT412, therefore 15% (v/v) glycerol was added to the medium prior to sterilization. After the cells had been removed by centrifugation, KT412 in the culture fluid was concentrated about 100-fold by ultrafiltration. This concentrate was dialysed, and subsequently resolved by cation-exchange chromatography

Table 1. Purification of the extracellular killer toxin, KT412, of *Z. bailii* 412

Purification step	Protein (mg)	Vol. (ml)	$10^{-6} \times$ Total activity (aU)	$10^{-3} \times$ Specific activity (aU mg ⁻¹)	Factor	Yield (% activity)
Culture fluid after ultrafiltration and dialysis	127	20	2.8	22	1	100
S-Sepharose	18	11	2.7	155	7	96
Superose TM ₁₂	2	1	1.8	880	40	64

Fig. 1. Purification of the killer toxin of *Z. bailii* 412 by cation-exchange chromatography (a) and gel filtration (b). ●, Killer toxin activity; —, protein; ---, NaCl gradient.

on S-Sepharose followed by gel filtration on Superose TM₁₂. The purification procedure is summarized in Table 1 and examples of column chromatograms are depicted in Fig. 1.

When the most active fraction obtained after gel filtration was subjected to SDS-PAGE, one protein band with a relative molecular mass of about 10 kDa was detected. The two bands in the range 60–70 kDa are due to an artefact and were probably caused by impurities in the mercaptoethanol preparation used. The SDS-PAGE of the preparations of KT412 during the course of purification is shown in Fig. 2. The apparent molecular mass of KT412 was confirmed by gel filtration on Superose TM₁₂. The killer toxin eluted between cytochrome *c* and aprotinin, indicating an apparent molecular mass of about 10 kDa.

KT412 could not be stained with PAS; this means that no carbohydrate moiety could be detected. The well-known glycoprotein toxin K₂₈ of *S. cerevisiae* gave a distinct signal. K₂₈ contains about 10 mannose residues per molecule (Schmitt & Pfeiffer, 1990). In accordance

Fig. 2. SDS-PAGE of preparations during the purification of KT412. Lane 1, low-molecular-mass markers; lane 2, aprotinin; lane 3, concentrated culture liquid of *Z. bailii*; lane 4, preparation after cation-exchange chromatography, fraction eluted before killer toxin activity; lane 5, KT412 after cation-exchange chromatography; lane 6, purified KT412 after gel filtration. Five micrograms of protein were applied to lanes 3 to 6.

with this observation KT412 did not bind to ConA-conjugated Sepharose. This can be regarded as further evidence that KT412 is not glycosylated.

Curing of the killer strain and investigation of its dsRNA plasmids

Killer yeasts can be cured of their killer phenotype by cultivation at elevated temperatures, or by treatment with cycloheximide or 5-fluorouracil (Fink & Styles, 1972; Mitchell *et al.*, 1973; Wickner, 1974; Zorg *et al.*, 1988). At first the attempts to cure *Z. bailii* 412 by growing at 35 °C, or treatment with ethidium bromide, cycloheximide, AZT, (3'-azido-3'-deoxythymidine), acridine orange or 5-fluorouracil were not successful. Then it was observed that in the presence of 10 mg cycloheximide l⁻¹ tiny colonies developed after about a week on YEP agar. When these colonies were propagated on YEP and then subcultured again on cycloheximide-containing YEP agar, it was found that about 3% of the

Fig. 3. Agarose gel electrophoresis of nucleic acid preparations of cured and killer strains of *Z. bailii*. Lane 1, λ -DNA (standard II); lane 2, *S. cerevisiae* 379 K2-killer; lane 3, *Z. bailii* 412; lanes 4 to 6, *Z. bailii* 412c (different cured strains); lane 7, *Z. bailii* 427 (non-killer).

survivors had lost their killer activity. Successful curing indicated that the killer character might depend on the presence of plasmids.

This was confirmed by isolating dsRNA from the wild-type *Z. bailii* 412, from three cured derivatives and, for comparison, from the K2-killer *S. cerevisiae* 379 that is known to possess two plasmids: L-dsRNA (3.8 kb) and M-dsRNA (1.5 kb). The nucleic acids were separated by agarose gel electrophoresis. (Fig. 3). L- and M-dsRNA of *S. cerevisiae* are clearly visible. The wild-type strain of *Z. bailii* 412 contained three plasmids. By comparison with the *EcoRI*/*HindIII* fragments of λ DNA the size of those plasmids was 4.0, 2.9 and 1.9 kb. When the extracts were treated with RNAase under low-salt conditions (120 μ g RNAase in 600 μ l of a solution of 10 mM-Tris + 10 mM-MgCl₂) or under high-salt conditions (additional 0.6 M-NaCl) at 37 °C for 1, 2 and 3 h the bands disappeared only under the low-salt conditions, indicating that they consisted of dsRNA.

The cured strains of *Z. bailii* that had lost the killer activity and the non-killer strain *Z. bailii* 427 showed only two bands (4.0 kb and 2.9 kb). In the extracts of the cured strains the amount of RNA of the 4.0 kb band increased. This increase of L-dsRNA copy number in the absence of M is a well-known phenomenon in cured strains of *S. cerevisiae* (Wickner, 1986).

Action and adsorption of KT412

The inhibition zones that appear in the lawn of the sensitive yeast (*S. cerevisiae* 452) around the wells in the agar diffusion test can be due to a microbicidal or a static action of the inhibiting agent. When killer toxin KT412 was added to growing yeast cells in YEPD medium, the microbicidal action was obvious (Fig. 4). Depending on the concentration of the killer toxin and on the sensitivity

Fig. 4. Influence of different concentrations of KT412 on the viability of two sensitive strains of *S. cerevisiae* (Δ, *S. cerevisiae* 381; ○, *S. cerevisiae* 452).

Fig. 5. Phase-contrast micrographs showing the effect of KT412 on the morphology of the sensitive strain *S. cerevisiae* 452. (a) Control; (b) with 27 aU KT412 ml⁻¹. Cells were incubated for 24 h at 20 °C in YEPD medium pH 4.0, with gentle shaking. Bars, 20 μ m.

of the tester strain, not only was growth inhibited but also cell viability decreased from about 10⁶ to 10² cells ml⁻¹ within 24 h.

The mode of action of KT412 on sensitive cells is unknown. The killer toxin K1 affects the proton gradient of the cell membrane and acts as an ionophore; thus low molecular mass solutes leak out of the cells, which finally die (Bussey, 1981). KT412 changed the appearance of a sensitive yeast at high concentrations. At the inhibitory but not lethal concentration of 27 aU about 1% giant cells were observed (Fig. 5). When treated with SDS, these round giant cells did not lyse, therefore it can be assumed that they are not spheroplasts lacking an intact cell wall.

According to the two-step model proposed for the action of killer toxin K1, the first step is the adsorption of the killer toxin to the cell wall (Bussey, 1981). Therefore, the adsorption of KT412 by yeast cells was tested. The killer toxin was added to different amounts of cells of the sensitive strain *S. cerevisiae* 452, the resistant strain *S. cerevisiae* 496 and the intermediate strain *S.*

Fig. 6. Adsorption of KT412 to resistant (●, strain 496) and sensitive strains (△, 381; ○, 452) of *S. cerevisiae* (100% residual activity corresponds to 155 aU KT412 ml⁻¹ after 1 h incubation at 4 °C in the absence of cells).

Table 2. Inactivation of KT412 by adsorption to the cell wall polysaccharides mannan and glucan of *S. cerevisiae* 452, and pustulan and laminarin

The sensitive strain *S. cerevisiae* 452 (5.4×10^5 c.f.u.) was incubated with 90 aU KT412 in 3 ml YEPC medium, pH 4, for 24 h at 20 °C with gentle shaking.

Polysaccharide	Concn (mg ml ⁻¹)	10 ⁻⁵ × Viable cells (c.f.u. ml ⁻¹)
Control	No killer toxin, no polysaccharide	346
	With killer toxin, no polysaccharide	2.5
Mannan	2.5	3.4
	5	17
	10	227
	15	256
	20	343
G1-glucan	5	2.6
	10	7.4
	15	7.3
	20	13
β-1,3-D-Glucan	10	1.5
	15	1.6
	20	2.2
Pustulan (β-1,6-D-glucan)	10	1.3
	15	1.1
	20	1.7
Laminarin (β-1,3-D-glucan)	10	1.2
	15	1.3
	20	1.3

cerevisiae 381. After incubation for 1 h at 4 °C no killer toxin had been adsorbed by the resistant strain, whereas 10^8 cells ml⁻¹ of the sensitive strain had completely adsorbed KT412. Only some of the killer toxin was removed by the less sensitive strain *S. cerevisiae* 381 (Fig. 6). Similar results were obtained with isolated yeast cell walls (data not shown).

The main components of the yeast cell wall are glucans and mannoproteins. Hutchins & Bussey (1983) observed that the killer toxin K1 was adsorbed by a β-1,6-glucan, whereas a 180 kDa mannoprotein was identified as the primary receptor for the K₂₈ killer toxin of *S. cerevisiae* (Schmitt & Radler, 1987, 1988). To determine the primary acceptor of the cell wall for KT412, several polysaccharides and cell wall preparations were used in the adsorption test. When 0.54×10^6 cells of the sensitive yeast *S. cerevisiae* 452 were exposed to 89 aU of KT412, about 50% of the cells were killed within 24 h. In the absence of killer toxin, the cells grew to a total of 34.6×10^6 cells (Table 2). Of the polysaccharides tested, only the crude mannan fraction prevented the action of the killer toxin. Therefore, it was concluded that the primary binding sites for killer toxin KT412 are located within the mannan and not within the glucan fraction of the yeast cell wall.

Discussion

Since the first observation of killer toxins within the species *Saccharomyces cerevisiae* (Bevan & Makower, 1963), this phenomenon has been observed in many yeast genera. The first killer yeast was designated K1 when it was realized that another strain, called K2, showed no cross immunity. In fact, these two types killed each other. Due to similar cross-reactions, the first more general attempt to classify killer yeasts of seven genera was made by Young & Yagiu (1978), who assigned arbitrary numbers to killer yeasts. The killer strain of *Zygosaccharomyces bailii* 412 described in this paper differs from the killer yeasts that are known so far. Although forming an active killer toxin, the spectrum of activity is rather small, being restricted to certain strains of *S. cerevisiae* and *Candida glabrata*. More detailed studies have revealed that KT412 is active against most strains of *Zygosaccharomyces* (S. Kaiser, personal communication).

Although the spectrum of activity of killer yeasts is easily determined when suitable tester strains are available, it seems more rational to distinguish killer yeasts according to the characteristics of the killer toxins that they produce. All known killer toxins are acidic proteins, with an isoelectric point of about pH 4. Most killer toxins have a molecular mass in the range 10–20 kDa. The only exception is the killer toxin of *Kluyveromyces lactis*, which consists of three polypeptide subunits (27.5, 30, and 99 kDa). The existence of subunits (K₁ or killer toxin of *Kluyveromyces*) is not the rule. One main difference may be that some killer toxins, such as K₂, K₂₈ or the killer toxin of *Kluyveromyces*, contain carbohydrate residues; the others are obviously unglycosylated polypeptides. Whereas the killer toxin K₂₈

contains about 10 mannose residues, no carbohydrate was detected in KT412 with PAS or by adsorption to ConA-Sepharose. However, neither method is sufficiently sensitive or specific to detect a very small amount of mannose.

The characteristics of the different killer toxins depend on the information stored in the nucleic acid and on post-translational modification, i.e. glycosylation. The information for the killer toxin of *K. lactis* is encoded by two DNA plasmids. The killer toxins K_1 , K_2 and K_{28} of *S. cerevisiae* are encoded by distinct dsRNAs that are encapsidated by protein capsomers. The killer toxins of *Hanseniaspora uvarum* (Zorg *et al.*, 1988) and *Z. bailii* 412 belong to this group. *Z. bailii* 412 contains three dsRNA plasmids. It is assumed that the largest (4.0 kb) and the smallest (1.9 kb) correspond to the L- and M-plasmids of *S. cerevisiae*. This was shown by curing, as strains of *Z. bailii* without the M-plasmid lost killer activity. Furthermore, it has been possible to transfer the M-plasmid of *Z. bailii* 412 to a strain of *S. cerevisiae* by transfection, thereby converting this strain to a killer strain (M. J. Schmitt, personal communication). The significance of the third dsRNA (2.9 kb) is unknown; it is also protein encapsidated (F. Neuhausen, personal communication) and will be called Z-plasmid (Z for *Zygosaccharomyces*).

Conclusive evidence exists that the killer toxin K_1 acts on the membrane of sensitive cells (de la Peña *et al.*, 1981; Martinac *et al.*, 1990). However, it is doubtful that all killer toxins have the same mode of action; for example, K_{28} of *S. cerevisiae* has been shown to cause an early inhibition of DNA synthesis (Schmitt *et al.*, 1989). With regard to the primary receptor of the cell wall, the killer toxins show different affinities. The killer toxins K_1 and K_2 of *S. cerevisiae* and that of *H. uvarum* are adsorbed by glucans, whereas killer toxin K_{28} and the killer toxins of *Debaryomyces hansenii* (S. Kilian, personal communication) and *Z. bailii* (this paper) are adsorbed by mannans of the yeast cell wall. As the latter two killer toxins are non-glycosylated polypeptides, the presence of mannose residues is obviously not essential for efficient binding to the yeast cell wall.

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