Curr Genet (1991) 19:389–393

Current Genetics

Linear DNA plasmids of *Pichia inositovora* are associated with a novel killer toxin activity

G. Thomas Hayman and Paul L. Bolen

Microbial Properties Research, National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, N. University Street Peoria, IL 61604, USA

Received October 27, 1990/January 14, 1991

Summary. *Pichia inositovora*, strain NRRL Y-18709, which contains three linear double-stranded DNA plasmids, p*Pin*l-1, p*Pin*l-2 and p*Pin*l-3, was cured of these plasmids both by growing the strain in the presence of 50 μg/ml bisbenzimide, and by exposure to ultraviolet light. Both cured and uncured strains were tested for growth on a variety of carbon sources. No differences in growth response were detected, indicating no discernible involvement of the linear plasmids in the catabolism of these compounds. Culture supernatants of *Pichia inositovora* were shown to contain a substance larger than 100 kDa that is toxic to *Saccharomyces cerevisiae*, strain GS 1688. Toxin activity was optimal in YEPD assay plates containing 50 mM citrate buffer with a pH between 3.4 and 4.2. Culture supernatants from *P. inositovora* were also weakly active against *Cephaloascus albidus*, strain NRRL Y-18710, and *Citeromyces matritensis*, strain NRRL Y-18711. Concentrated supernatants from cured *P. inositovora* strains did not exhibit these activities, consistent with the hypothesis that this toxic activity is linear plasmid-encoded. Unlike the well-known *Kluyveromyces lactis* system, or the newly identified *P. acaciae* system, *P. inositovora* strains cured of their linear plasmids do not become detectably sensitive to toxin produced by the wild-type strain, suggesting a nonplasmid-encoded immunity function.

Key words: Yeast – *Pichia inositovora* – Linear plasmids – Killer toxin

Introduction

Numerous filamentous fungi and yeasts have been found to contain linear double-stranded DNA plasmids (Meinhardt et al. 1990; Samac and Leong 1989 and references within). Among the yeasts that contain these DNA elements are *Saccharomyces kluyveri* (Kitada and Hishinuma 1987), *Pichia acaciae* (Worsham and Bolen 1990), *Kluyveromyces lactis* (Stark et al. 1990 and references within), *Phaffia rhodozyma* (Wilber and Proffitt 1987), *Saccharomycopsis crataegensis* (Shepherd et al. 1987) and *Pichia inositovora* (Ligon et al. 1989). Although most fungal linear plasmids are located in the mitochondria (Samac and Leong 1989), the yeasts *K. lactis*, *S. crataegensis*, and *P. inositovora* have these elements in the cytoplasm. Based on shared characteristics with other linear DNA plasmids, such as 5′ ends protected by proteins and terminal inverted repeats, these cytoplasmic episomes have been included in a class of DNA molecules with the proposed name "invertrons" (Sakaguchi 1990).

To date, functions have been ascribed to linear DNA plasmids of yeasts in only two cases. The most completely studied is the *K. lactis* system, consisting of two plasmids, pGKL1 and pGKL2, which are 8.1 and 13.4 kilobases (kb) in size, respectively. The smaller of the two plasmids, pGKL1, has been shown to encode both production of and immunity to a three subunit proteinaceous toxin active against a wide range of yeast species (Stark et al. 1990; Gunge et al. 1981). More recently, an analogous plasmid system has been discovered in *P. acaciae*, strain NRRL Y-18665 (Worsham and Bolen 1990). As with *K. lactis*, this strain contains two linear DNA plasmids, and the smaller of the two, p*Pac*l-2, appears to encode a proteinaceous, heat-labile toxin, as well as immunity to this toxin (P. L. Worsham and P. L. Bolen, unpublished experiments). A function has not yet been discovered for the linear DNA plasmids of the yeasts *S. kluyveri* and *S. crataegensis*. This report describes the identification and initial characterization of a novel killer toxin associated with the linear plasmids of *P. inositovora* strain NRRL Y-18709.

Materials and methods

Strains and media. Yeast strains were grown using YEPD (1.0% yeast extract, 2.0% Bacto-peptone, 2.0% glucose), buffered when required with 50 mM sodium citrate buffer at the appropriate pH. The minimal medium used was YNB (Difco; containing 0.5% am-

Offprint requests to: P. L. Bolen

monium sulfate and 2.0% glucose or other carbon source). Solid media contained 1.5% agar. All cultures were normally grown at 25 °C.

Plasmid curing. Yeast strains were cured of their linear DNA plasmids both by growing sequential 3- to 4-day YEPD cultures containing 50 µg/ml bisbenzimide with several transfers at 25 °C and 29 °C, and by exposure to ultraviolet light (UV; 254 nm) at a level sufficient to kill approximately 80% of the cells, essentially as described by Worsham and Goldman (1988).

Plasmid isolation and electrophoresis. Plasmids were isolated and identified by agarose gel electrophoresis using previously described procedures (Ligon 1989).

Electroelution and Southern hybridization. Electroelution and radiolabeling of DNA probes, and hybridization analyses, were carried out as described by Ligon et al. (1989).

Toxin bioassays. Killer toxin activity present in culture supernatants was assayed using a modification of the method of Worsham and Bolen (1990). Cells were harvested from 1 or 2 l YEPD cultures by centrifugation at 3000 g for 10 min. Culture supernatants were concentrated 500- to 1000-fold using Amicon 52 and 8400 ultrafiltration units fitted with YM100 filters (100000 MW cut-off), operated under 20 psi N_2 at room temperature. Concentrated supernatants were filter-sterilized and stored at 4 °C. Aliquots of these culture concentrates (5 µl) were applied to wells of a 24-well plastic microtiter dish, each well containing 1 ml citrate-buffered YEPD agar, and allowed to dry. Molten top agar (0.5 ml; YEPD plus 0.7% agar) held at 50 °C was inoculated with 30 µl of an overnight culture of an indicator strain which had been diluted to an A_{600} of approximately 0.7. The top agar was then mixed and overlaid on the agar in each well. Toxin production was assayed by patching strains to be tested on a YEPD plate containing 50 mM citrate buffer (pH 3.6) and allowing them to grow for 2–3 days. The grown patches of cells were then killed by exposure to chloroform vapor for 10 min, followed by airing for 5 min. The plate was then overlaid with top agar seeded with *S. cerevisiae* strain GS 1688 and incubated for 24 to 48 h at 25 °C.

Results

Isolation of cured strains

Cultures from single-colony isolates of *P. inositovora* strain NRRL Y-18709, either grown at 25 °C or 29 °C in the presence of bisbenzimide or else subjected to UV light, were used to isolate plasmid DNA. Of 148 colonies analyzed from bisbenzimide-treated cultures, five were found to contain no detectable plasmid DNA. Five others lacked p*Pin*l-2, and one harbored p*Pin*l-1, p*Pin*l-3 and what appeared to be p*Pin*l-2 with a deletion. The remaining strains contained all three linear plasmids. Of 28 isolates from a UV-treated culture, only three retained detectable plasmid DNA. These three strains harbored p*Pin*l-1 and p*Pin*l-3, and appeared to contain p*Pin*l-2 at lower levels than the wild-type strain. Plasmid DNA patterns from the four classes obtained are shown in Fig. 1. Plasmid preparations from the wild-type and cured strains isolated in the absence of RNase showed no distinguishable RNA plasmid species (data not shown).

Southern hybridization

It has been demonstrated that there is no detectable hybridization between the three p*Pin*l plasmids or between these episomes and the *S. cerevisiae* 2 µm plasmid, the three linear plasmids of *S. crataegensis*, or the two *K. lactis* linear plasmids (Ligon et al. 1989). The three *P. inositovora* plasmids also showed no detectable homology to either of the two linear plasmids of *P. acaciae* (data not shown).

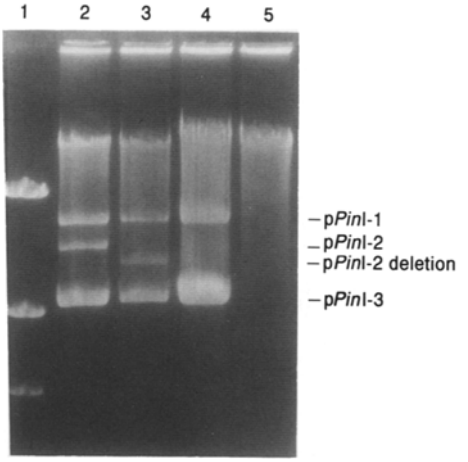

Fig. 1. Linear plasmids of selected *P. inositovora* strains. Plasmid DNA was isolated from 25 ml cultures, subjected to 0.8% agarose gel electrophoresis and stained with ethidium bromide. *Lane 1,* λ/*Hin*dIII; *Lane 2,* NRRL Y-18709 (wild-type); *Lane 3,* GS 931 (p*Pin*l-2 deletion); *lane 4,* GS 929 (cured of p*Pin*l-2); *lane 5,* GS 1004 (cured). Plasmid identities are given at the side of the figure

To determine whether p*Pin*l-2 had suffered a deletion in strain GS 931 and had been cured in strain GS 929, hybridizations of a blot made from a gel identical to that shown in Fig. 1 were performed using electroeluted, ^{32}P-labeled p*Pin*l-2 DNA as a probe. p*Pin*l-2 of wild-type strain Y-18709 and the putative p*Pin*l-2 deletion plasmid of strain GS 931 hybridized strongly to the probe, indicating that the latter plasmid is in fact a deletion derivative of p*Pin*l-2. No hybridization signal was detected between the positions of p*Pin*l-1 and p*Pin*l-3 of strain GS 929, indicating that this isolate is completely cured of p*Pin*l-2 (data not shown).

Assimilation studies

The wild-type *P. inositovora* strain and a fully cured derivative were tested for ability to grow on YNB minimal solid media containing different carbon compounds as the sole carbon and energy source. Both strains grew well on all compounds tested, except lactic acid, L-arabinose, D-ribose, and D-glucosamine. The latter three compounds supported slight growth of both isolates. Lactic acid was not utilized by either strain (Table 1). Our observation that *P. inositovora* strain NRRL Y-18709 exhibits minimal growth on media containing L-arabinose, D-ribose and D-glucosamine differs slightly from previous reports (Barnett et al. 1983; C. P. Kurtzman, personal communication).

Identification of a new killer toxin

Filter-sterilized concentrated culture supernatants of both wild-type and cured *P. inositovora* strains were used

Table 1. Utilization of different carbon compounds by wild-type and cured *Pichia inositovora* isolates[a]

	L-arabinose	Cellobiose	Galactose	Maltose	Melezitose	D-ribose	Sorbose	Sucrose	D-xylose	D-glucosamine	α-methyl glucoside	Ethanol	D-glucitol	D-glycerol	Myo-inositol	D-mannitol	Ribitol	Citric acid	D-glucuronic acid	Lactic acid	Succinic acid
NRRL Y-18709 (wild-type)	±	+	+	+	+	±	+	+	+	±	+	+	+	+	+	+	+	+	+	−	+
GS 1004 (cured)	±	+	+	+	+	±	+	+	+	±	+	+	+	+	+	+	+	+	+	−	+

[a] +, able to use the indicated compound as sole source of carbon and energy; −, unable to metabolize; ±, detectable growth, intermediate between growth on media containing dextrose and on media lacking a carbon source

Table 2. Reactions of different *Saccharomyces cerevisiae* strains to *Pichia inositovora* isolates[a]

Strain	Genotype	Toxin reaction[a]	Origin
GS 1688	*MATα ade his* ϱ^0	S	P. Perlman
GS 1731	*MATα ade his* ϱ^+	S	P. Perlman
GS 1725	*MATa leu2 ura3 trp1 ade2 his3* ϱ^0	R	A. Myers
GS 643	*MATa leu2 ura3 trp1 ade2 his3* ϱ^+	R	A. Myers

[a] R, resistant to concentrated culture supernatants of strains NRRL Y-18709 and GS 1004 in a microtiter dish assay; S, sensitive to concentrated culture supernatants of strain NRRL Y-18709 while resistant to strain GS 1004 culture supernatant concentrates

to screen yeast isolates for toxin sensitivity using a microtiter dish plate bioassay. The wild-type strain, three bisbenzimide-cured and 16 UV-cured *P. inositovora* isolates were all found to be insensitive to culture supernatants of both the wild-type strain and a cured isolate. However, two genetically similar strains of *S. cerevisiae*, GS 1688 and GS 1731, were discovered to be very sensitive to wild-type culture supernatants, but insensitive to sterile culture concentrates of the cured strain (Fig. 2, Table 2, data not shown). Subsequently, two other yeast species, *Cephaloascus albidus*, strain NRRL Y-18710, and *Citeromyces matritensis*, strain NRRL Y-18711, were shown to be slightly sensitive to culture supernatants of the wild-type but not of the cured strain (Fig. 2).

Toxin characterization

To determine the optimum pH for toxin activity, the microtiter dish assay was used, with strain GS 1688 as the indicator. The pH of the citrate buffer added to the agar in each well was varied, by increments of 0.2, from pH 3.0 to 6.0. As shown in Fig. 3, toxin activity was strongest when citrate buffers of pH 3.4 to 4.2 were used.

Exposure of the toxin in culture concentrates to 65 °C for 10 min resulted in complete loss of activity against strain GS 1688. Incubation of toxin in proteinase K buffer (Sambrook et al. 1989), lacking sodium dodecyl

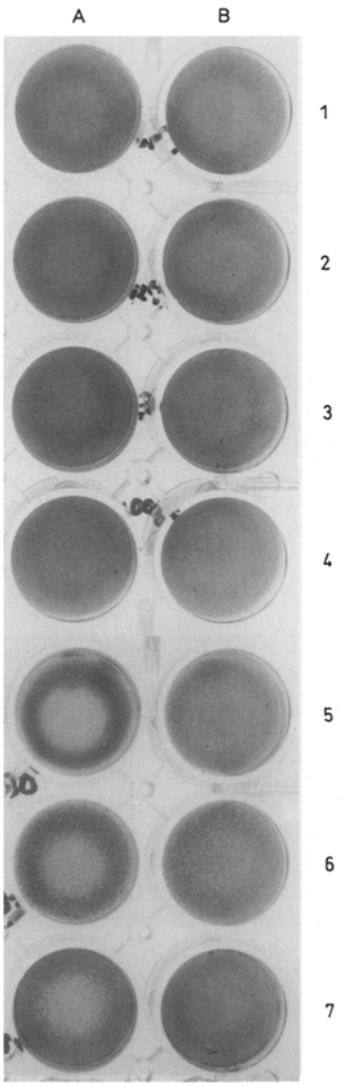

Fig. 2. Bioassay of *P. inositovora* killer toxin. Assays were performed as described in Materials and methods. Strains from which culture supernatants were concentrated and spotted on the plates are: **A** NRRL Y-18709 (*P. inositovora* wild-type); **B** GS 1004 (*P. inositovora* cured). Indicator strains used in the overlays are numbered to the right of each pair of wells: *1*, NRRL Y-18709 (*P. inositovora* wild-type); *2*, GS 931 (*P. inositovora* p*Pin*l-2 deletion); *3* GS 929 (*P. inositovora* cured p*Pin*l-2); *4*, GS 1004 (*P. inositovora* cured); *5*, GS 1688 (*S. cerevisiae*); *6*, NRRL Y-18711 (*Citeromyces matritensis*); *7*, NRRL Y-18710 (*Cephaloascus albidus*)

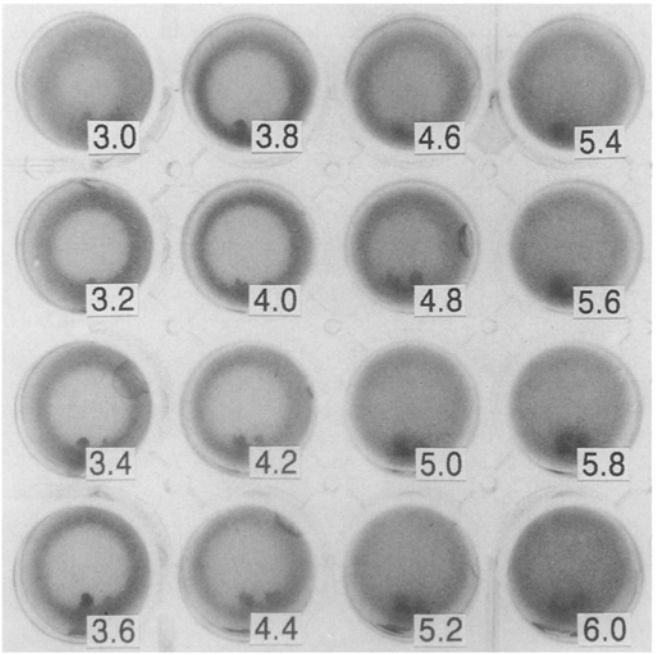

Fig. 3. Optimum pH assay for *P. inositovora* killer toxin activity. Citrate buffer (50 mM) of the pH indicated has been added to the YEPD solid medium in each well. Concentrated culture supernatant aliquots from strain NRRL Y-18709 were spotted on the agar in all wells. The indicator strain used to seed the overlays was strain GS 1688

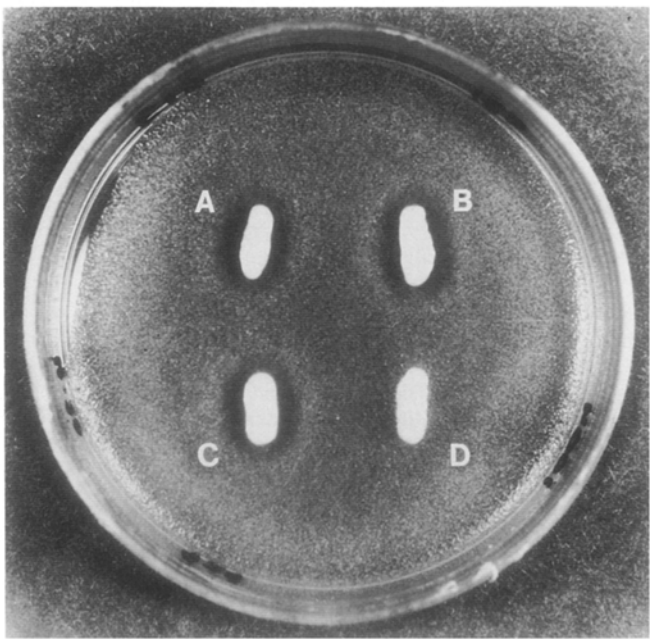

Fig. 4. Toxin production by selected *P. inositovora* strains. Strains to be tested were patched onto a YEPD-citrate pH 3.6 plate and grown overnight: *A*, NRRL Y-18709 (wild-type); *B*, GS 931 (p*Pin*l-2 deletion); *C*, GS 929 (cured of p*Pin*l-2) *D*, GS 1004 (cured). The grown patches were killed by exposure to chloroform vapor, and the plate was overlaid with top agar seeded with sensitive indicator strain GS 1688, followed by incubation at 25 °C

sulfate and containing 1 mg/ml proteinase K, at 37 °C for 2 h significantly reduced activity, compared to incubation of the toxin in the presence of proteinase K buffer alone, which had no effect (data not shown).

Toxin sensitivity vs the rho (ϱ) phenotype

As *S. cerevisiae*, strain GS 1688, a ϱ^0 strain, was the first yeast discovered to be sensitive to the *P. inositovora* toxin, other ϱ^0 and ϱ^+ *S. cerevisiae* strains were examined for their reaction. Strain GS 1731, which is isogenic with strain GS 1688 except for its ϱ^+ character, was shown to be sensitive to the toxin, although slightly less so than strain GS 1688 (Table 2, data not shown). In contrast, neither of the *S. cerevisiae* strains, GS 643 (ϱ^+) nor GS 1725 (ϱ^0), isogenic except for their ϱ characters, showed any detectable reaction to the *P. inositovora* toxin (Table 2).

Toxin poduction

Production of toxin by *P. inositovora* isolates was assessed by the plate bioassay described in Materials and methods. Strains NRRL Y-18709 (wild-type), GS 931 (p*Pin*l-2 deletion), GS 929 (cured of p*Pin*l-2) and GS 1004 (fully cured) were patched onto a YEPD citrate (pH 3.6) plate and overlaid with top agar seeded with indicator strain GS 1688. Following incubation, clear zones were found around the patches of growth for all strains except the fully cured strain, GS 1004 (Fig. 4). The 28 UV-treated *P. inositovora* isolates analyzed for plasmid content were also screened for toxin production in the plate bioassay. The only three strains that tested positive were the same three found to contain both p*Pin*l-1 and p*Pin*l-3.

Discussion

The concentration of culture supernatants up to 1000-fold facilitated the discovery of a previously unidentified killer toxin of *P. inositovora*. Concentrated culture-medium components might prove toxic to some organisms, and be mistaken for toxin activity. For this reason, concentrated supernatants of a cured *P. inositovora* strain were analyzed in parallel as negative controls. Toxin concentration was not necessary to demonstrate activity, however, as demonstrated in Fig. 4.

Proteinaceous components of concentrated culture media present in toxin preparations may protect the toxin from proteinase K digestion, and may be responsible for the incomplete inactivation of the toxin by that enzyme. Alternatively, the *P. inositovora* toxin may be a glycoprotein, like the *K. lactis* toxin (Stark et al. 1990). Such a glycoprotein may possess carbohydrate moieties that interfere with proteinase K digestion.

Pichia inositovora appears similar to *P. acaciae* and *K. lactis* in that all three yeasts contain linear plasmids that encode, or apparently encode, a heat-labile, proteinaceous toxin active against other yeasts. Linear DNA plasmid-encoded killer toxins seem to be a repeated

theme in the ascomycetes. However, there appear to be three major differences between the killer toxin system in *P. inositovora* and the linear plasmid-associated toxin systems previously characterized in *K. lactis* and *P. acaciae*. First, *P. inositovora* contains a third linear plasmid while there are only two detected in *P. acaciae* and *K. lactis*. This third plasmid, p*Pin*l-2, does not appear to be involved with the *P. inositovora* toxin system, as its loss has no effect on toxin production or toxin sensitivity (Figs. 2 and 4). Second, the *P. inositovora* toxin has a different spectrum of activity. Of yeasts sensitive to the *K. lactis* and *P. acaciae* toxins, only four strains have so far been found that are sensitive to the toxin of *P. inositovora* and, in two of these, sensitivity is low. The *P. acaciae* and *K. lactis* killer toxins affect a wide range of yeast genera (Gunge et al. 1981; G. T. Hayman and P. L. Bolen, unpublished data); the range of the *P. inositovora* toxin is presently being determined. Last, unlike the *P. acaciae* and *K. lactis* toxin system, in which there is a linear plasmid-associated immunity system (Stark et al. 1990; Worsham and Bolen 1990), there appears to be no such protective function associated with the linear plasmids of *P. inositovora*. Such a function may, however, be chromosomally encoded. Alternatively, *P. inositovora* may possess a linear plasmid-encoded immunity function, but may in addition be inherently resistant to its own toxin by virtue of a nonlinear plasmid-determined, presumably chromosome-encoded, resistance. This would make such an immunity determinant difficult to detect. Immunity is readily observed in *P. acaciae* because it becomes very sensitive to its own toxin when cured of its linear plasmids. However, *K. lactis* strains cured of their plasmids become only slightly sensitive to *K. lactis* toxin (M. J. R. Stark, personal communication; our unpublished observations). Cured strains of *P. inositovora* may be even less sensitive to toxin produced by the wild-type strain.

The first strain discovered to be sensitive to a product of *P. inositovora*, strain NRRL Y-18709, was the ϱ^0 *S. cerevisiae* strain, GS 1688. There is an experimental precedent for an, as yet, uncharacterized incompatibility between *K. lactis* linear plasmids and ϱ^+ strains of *S. cerevisiae*; the linear plasmids appear to be able to replicate only in ϱ^0 strains of this yeast species (Gunge and Yamani 1984). At first it seemed possible that there was a connection between the observed incompatibility of linear plasmids and mitochondrial DNA, and our finding that a ϱ^0 *S. cerevisiae* strain was sensitive to a product of a linear plasmid-containing yeast. That the product of *P. inositovora*, strain NRRL Y-18709, is a true killer toxin, and not merely a metabolite toxic only to ϱ^0 strains, is supported by the following observations: (1) a ϱ^+ strain of *S. cerevisiae*, GS 1731, isogenic with strain GS 1688 except for its ϱ character, is also sensitive to strain NRRL Y-18709 culture concentrates, (2) another ϱ^0 strain, GS 1725, and its counterpart GS 643, isogenic except for the ϱ trait, are both resistant to culture supernatants of strain NRRL Y-18709, and 3) such supernatants are also active, albeit weakly, against two other wild-type yeast species (see Fig. 2 and Table 2). Purification of the *P. inositovora* toxin is underway, which will allow further comparisons between the three known linear DNA plasmid-associated yeast killer toxins.

Acknowledgements. The authors wish to thank D. A. McCracken and M. J. R. Stark for helpful discussions; N. J. Alexander for providing strains GS 1688 and GS 1731, originally from P. Perlman; A. Myers for providing strains GS 643 and GS 1725; B. M. Mannarelli and H. S. Shepherd for critically reviewing the manuscript, and S. L. Tulloh, W. L. Orton, J. S. Stott, and P. L. Cihak for expert technical assistance. This research was supported by Biotechnology Research and Development Corporation project grant No. 1-3-010 to P. L. Bolen.

References

Barnett JA, Payne RW, Yarrow D (1983) Yeasts: characteristics and identification. Cambridge University Press, Cambridge

Gunge N, Tamaru A, Ozawa F, Sakaguchi K (1981) J Bacteriol 145:382–390

Gunge N, Yamani C (1984) J Bacteriol 159:533–539

Kitada K, Hishinuma F (1987) Mol Gen Genet 206:377–381

Ligon JM, Bolen PL, Hill DS, Bothast RJ, Kurtzman CP (1989) Plasmid 21:185–194

Meinhardt F, Kempken F, Kämper J, Esser K (1990) Curr Genet 17:89–95

Sakaguchi K (1990) Microbiol Rev 54:66–74

Samac DA, Leong SA (1989) Mol Plant-Microbe Interact 2:155–159

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Shepherd HS, Ligon JM, Bolen PL, Kurtzman CP (1987) Curr Genet 12:297–304

Stark MJR, Boyd A, Mileham AJ, Romanos MA (1990) Yeast 6:1–29

Wilber KA; Proffitt JH (1987) Yeast Genetics and Molecular Biology Meeting, June 16–21, Genetics Society of America, San Francisco, California, Abstract 106

Worsham PL, Bolen PL (1990) Curr Genet 18:77–80

Worsham PL, Goldman WE (1988) Mol Gen Genet 214:348–352

Communicated by K. Wolf