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Killer toxin production in *Pichia acaciae* is associated with linear DNA plasmids

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Summary. We have identified a strain of the yeast *Pichia acaciae* which produces a "killer" toxin active against the yeast *Debaryomyces tamarii*. The killer phenotype was associated with the presence of two DNA plasmids, p*Pac*l-1 (13.6 kilobase pairs) and p*Pac*l-2 (7.3 kilobase pairs). *P. acaciae* strains, cured of these plasmids by irradiation with ultraviolet light, lacked killer activity and were sensitive to toxin produced by the parental strain. A partially cured strain, GS-1215, missing only the smaller plasmid, p*Pac*l-2, also exhibited loss of both toxin activ ity and immunity. Exonuclease studies revealed that both plasmids were linear double-stranded DNA molecules with 5′ protected ends. The *P. acaciae* system differs from that of the well-studied *Kluyveromyces lactis* "killer" system both in the range of susceptible strains and in the sizes of the plasmids involved. Our studies contradict previous reports that *Pichia* killer systems are invariably chromosomal.

Key words: Yeast *Pichia acaciae* – Killer toxin – Extrachromosomal DNA

Introduction

A number of yeasts have been shown to carry linear double-stranded DNA plasmids. Most of these plasmids are cryptic, i.e., curing does not lead to observable changes in phenotype when the cured strains are grown under laboratory conditions (Shepherd et al. 1987; Kitada and Hishinuma 1987; Ligon et al. 1989). Only the linear plasmids pGKL1 and pGKL2 of *Kluyveromyces lactis* have known functions; these plasmids are responsible for the production of a "killer" toxin, an antifungal protein active against a number of yeast species (Gunge et al. 1981; Gunge 1986; Lehman et al. 1987). The smaller plasmid, pGKL1, encodes both a killer toxin and immunity to the toxin, while the larger plasmid, pGKL2, appears to encode replicative functions necessary for both plasmids. There is evidence that pGKL2 may also have some function in immunity (Tokunaga et al. 1987).

Our laboratory has screened a number of yeast strains for the presence of linear DNA plasmids. An analysis of plasmid-bearing strains revealed that a strain of *Pichia acaciae* possessed two linear plasmids and inhibited the growth of *Debaryomyces tamarii* when the two strains were cross-streaked. In this report, we describe a killer toxin activity and immunity which appear to be associated with the linear plasmids of *P. acaciae*. We also present evidence that these toxin and immunity functions could be encoded by the smaller of the two linear plasmids carried by *P. acaciae*.

Materials and methods

Strains and culture conditions. P. acaciae (NRRL Y-18665) and *Debaryomyces tamarii* (NRRL Y-18666) strains were obtained from the ARS Culture Collection, Northern Regional Research Center, Peoria Ill. All cultures were grown at 25 °C in YPD broth (10 g yeast extract, 20 g peptone, 20 g dextrose/l) with vigorous shaking (200 RPM) or on solid YPD medium (15 g agar/l). Toxin assay medium was 10% glycerol, 50 mM citrate buffer (pH 5.2), 1% yeast extract, 2% peptone, 2% dextrose, 30 μg/ml methylene blue, and 16 g/l Difco agar.

Plasmid nomenclature. To name the linear plasmids identified in this study, we have followed the system set forth in Shepherd et al. (1987) and Ligon et al. (1989). Following a lower case p (plasmid), three underlined letters were drawn from the genus (capitalized first letter) and species (lower case first and second letters) names. These letters were followed by a lower case l (linear), a dash, and a number to indicate the particular molecular species; numbering from the highest molecular weight species to the lowest. This methodology assures a unique indicator for each plasmid, designates them as linear plasmids, and allows some recognition of the particular yeast in which they were discovered.

Preparation of yeast plasmid DNA. Overnight cultures (3 ml) of *P. acaciae* strain NRRL Y-18665 were harvested by centrifugation. The cell pellet was washed with PE buffer (0.1 M sodium phos-

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phate, 0.1 M EDTA, pH 7.0) and resuspended in 800 µl of the same. Protoplasts were prepared by the addition of 50 µl of freshly prepared zymolyase (10 mg/ml, aqueous) followed by a 2 h incubation at 37 °C. Protoplasts were collected by centrifugation in an IEC clinical tabletop centrifuge (approx. 625 g), resuspended in 1 ml of PES buffer (50 mM sodium phosphate, 0.1 M EDTA, 50 mM NaCl) and lysed by the addition of 50 µl of 20% sarcosyl. Lysates were treated with RNase A (final concentration 1 mg/ml) and proteinase K (final concentration 0.5 mg/ml) for 2 h at 37 °C before removing cell debris by centrifugation. Cleared supernatants were then passed through a column of glass wool to decrease the amount of high molecular weight DNA in each preparation. One-quarter volume of 5 M NaCl was added to each sample and these were stored overnight at 4 °C. Plasmid preparations were then extracted three times with phenol saturated with 0.1 M TRIS, pH 8.0/chloroform/isoamyl alcohol (24:23:1) and twice with chloroform/isoamyl alcohol (24:1) prior to ethanol precipitation (Maniatis et al. 1982). The pellets were washed with ice-cold 70% ethanol and absolute ethanol and dried. They were resuspended in TES with 50 µg/ml RNase A, and incubated at 65 °C for 10 min, followed by an incubation at 37 °C for 1 h. Preparations were stored at 4 °C.

Analysis of DNA. Agarose gel electrophoresis was performed by conventional methods (Maniatis et al. 1982). Reactions with restriction endonucleases, lambda exonuclease, exonuclease III, DNase, RNase A, and proteinase K were performed according to manufacturers' instructions. *Hind*III fragments of bacteriophage lambda were used as molecular size standards.

Toxin assay. Toxin activity was assayed by a modification of previously described methods (Lehmann et al. 1987; Starmer et al. 1987). Approximately 10^4 cells of the indicator strain were seeded into 20 ml of molten toxin assay agar (approximately 45 °C) which was then poured into sterile petri dishes. After the plates solidified, wells (approximately 6 mm in diameter) were punched into the agar surface using the large end of a sterile pasteur pipet. The agar plug was removed aseptically and discarded. The wells were then filled with 65 µl of the material to be tested for toxin activity and the plates were incubated, agar-side down, for 48 h at 25 °C. A zone of growth inhibition around the well was interpreted as a positive test.

Preparation of culture supernatants for toxin assay. Overnight cultures of *P. acaciae* were grown in YPD at 29 °C and harvested by centrifugation at 5000 g. Supernatants were buffered with 50 mM citrate, pH 5.2, stored at 4 °C, and used the same day.

U.V. curing of plasmids. An exponentially growing culture of *P. acaciae* was diluted to 10^7 cells/ml in sterile saline and irradiated as described previously (Worsham and Goldman 1988). Cultures were diluted and plated on YPD medium to determine viability before and after irradiation.

Results

Assay of killer toxin produced by P. acaciae

Previously published procedures were modified to optimize determinations of *P. acaciae* toxin activity. The bioassay described herein is reproducible and relatively simple to perform.

D. tamarii, NRRL Y-18666, served as the indicator strain for our experiments since zones of inhibition were not observed when other yeast, including strains of *Saccharomyces cerevisiae* and *Saccharomycopsis crataegensis*, were used as indicators. The latter two strains are sensitive to *K. lactis* killer toxin (P. L. Bolen and J. M. Ligon, unpublished observations). The growth medium chosen (see Materials and methods) is conducive to rapid growth of the indicator strain and maximizes toxin activity.

Optimum pH for the assay was determined by comparing toxin activity at various pH values. Both the assay medium and the buffer of the culture supernatants were adjusted to the appropriate pH with 50 mM citrate buffer. Activity was observable from pH 4.75–5.60; the pH optimum, as judged by the diameter of the zone of inhibition, was approximately 5.2. No growth inhibition was observed at pH 4.15; the indicator strain did not grow at pH 6.0.

Growth conditions of the toxin producer also influenced assay results. The highest level of activity was found in culture supernatants from cultures grown at 29 °C. Detectable toxin activity was not found in culture fluids of yeasts grown at 37 °C.

Characterization of plasmids in P. acaciae

Two plasmids were identified in *P. acaciae* strain NRR Y-18665: p*Pac*l-1 (13.6 kb) and p*Pac*l-2 (7.3 kb). These plasmids were insensitive to RNase A, but sensitive to DNase. Exonuclease studies revealed that both plasmids were sensitive to the 3′-specific exonuclease III, but resistant to lambda exonuclease, which acts on 5′ ends (Fig. 1). These data suggest that these plasmids, like others identified in our laboratory, are double-stranded linear DNA molecules with protected 5′ ends (Shepherd et al. 1987; Ligon et al. 1988).

Association of toxin production and immunity with linear plasmids

When *P. acaciae*, NRRL Y-18665, was irradiated with UV light (93% killing), approximately 58% (110/190) of the survivors lacked both plasmids. A total of 40 randomly chosen colonies were tested for toxin activity and sensitivity to toxin produced by the parental strain. In each case tested, cured strains had lost toxin activity (Tox^-) and were sensitive to the toxin of the parental strain (Imm^-). All strains retaining both plasmids were Tox^+ and Imm^+. In subsequent experiments, one partially cured strain was identified which retained only the larger of the two plasmids (Fig. 2, lane 7). This strain, GS-1215, like comparably cured strains of *K. lactis*, was Tox^- Imm^-. Only one partially cured strain was identified. Loss of the Tox^+ phenotype appeared to be irreversible; none of the Tox^- strains ever regained the ability to make toxin, even after numerous transfers to fresh media.

We also attempted to cure *P. acaciae*, NRRL Y-18665, of the linear plasmids by growth at 37 °C, because we suspected that the lack of toxin activity in supernatants from 37 °C cultures might be the result of plasmid curing at this temperature. However, plasmid preparations from *P. acaciae* yeasts grown at 37 °C still carried both plasmids (data not shown). Furthermore, strains repeatedly subcultured at 37 °C regained the Tox^+ phenotype when grown at lower temperatures. Thus, growth at high temperature may affect production or stability of the toxin *per se* (Radler et al. 1985).

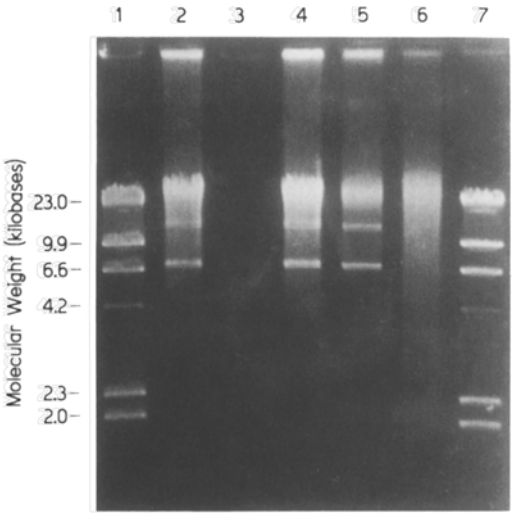

Fig. 1. Electrophoretic separation of nuclease-treated plasmid-containing nucleic acid preparations from *Pichia acaciae* strain NRRL Y-18665. *Lanes 1 and 7* contain molecular mass markers of bacteriophage lambda DNA fragments prepared by digestion with the restriction endonuclease *Hind* III. Portions of a plasmid-containing nucleic acid preparation from NRRL strain Y-18665 are shown following no treatment (*lane 2*), digestion with DNase I (*lane 3*), digestion with RNase (*lane 4*), digestion with lambda exonuclease (*lane 5*) and with exonuclease III (*lane 6*)

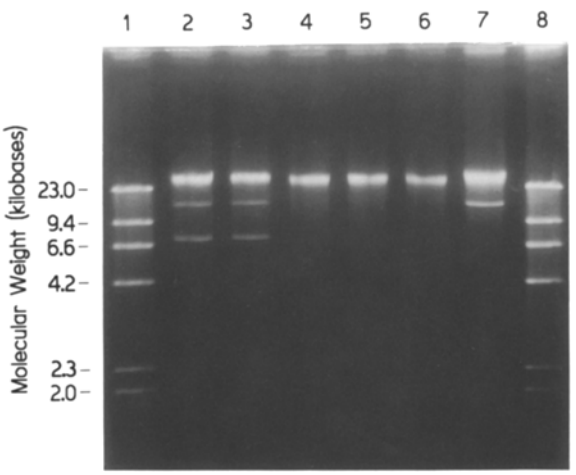

Fig. 2. Electrophoretic separations of nucleic acid preparations from wild-type and ultraviolet light (UV)-treated isolates of *Pichia acaciae* strain NRRL Y-18665. *Lanes 1 and 8* contain molecular size markers of bacteriophage lambda DNA fragments prepared by digestion with the restriction endonuclease *Hind* III. Nucleic acid preparations are shown from NRRL strain Y-7773 (*lane 2*), GS-1035, Tox^+ Imm^+ (*lane 3*), GS-1037, Tox^- Imm^- (*lane 4*), GS-1038, Tox^- Imm^- (*lane 5*), GS-1039, Tox^- Imm^- (*lane 6*) and GS-1215, Tox^- Imm^- (*lane 7*)

Discussion

Killer toxins have been found to be encoded by chromosomal genes (Starmer et al. 1987), DNA plasmids (Gunge 1986) and RNA plasmids (Wickner 1976). Previously reported toxins produced by the genus *Pichia*, such as those described in *P. kluyveri* and *P. anomala*, are apparently chromosomal (Starmer et al. 1987; Sawant et al. 1988; Zorg et al. 1988). In *P. acaciae*, NRRL Y-18665, however, it appears that the killer system more closely resembles that of *K. lactis*, in which toxin production and immunity are both associated with linear double-stranded DNA molecules. Although we did not confirm curing by Southern hybridization, the association between loss of toxin activity and the change in plasmid profile is clear. It is possible that the plasmids might still be present in a much lower copy number or integrated into the chromosome. If so, these changes in plasmid number or location are sufficient to inhibit detectable production of toxin and the expression of immunity.

The Tox^- Imm^- phenotype of the partially cured strain GS-1215 suggests that both toxin production and expression of immunity are dependent on the smaller of the two linear plasmids, p*Pac*l-1. We cannot be certain at this time, however, that the change in phenotype is not due to ultraviolet light-induced mutations in p*Pac*l-1. Confirmation of the location of the toxin and immunity genes awaits further experiments, e.g., transformation of p*Pac*l-2 into the partially cured strain GS-1215. These studies, however, will require the development of a genetic transformation system for this organism.

Like linear plasmids in other yeasts and filamentous fungi, the linear plasmids of *P. acaciae* appear to have 5′ termini which are resistant to lambda exonuclease digestion (Meinhardt et al. 1986). Proteins have been identified that are attached to the 5′ termini of the *K. lactis* linear plasmids and likely lead to this exonuclease protection. These 5′ proteins are throught to be involved in plasmid replication (Stam et al. 1986).

The correlation between the Tox^+ Imm^+ phenotype and the presence of the smaller of two *P. acaciae* linear DNA plasmids is reminiscent of the *K. lactis* killer system. Indeed, the failure to identify strains which contain only p*Pac*l-2 can be readily explained if p*Pac*l-1, like its counterpart pGKL2 in *K. lactis*, is required for replication of the smaller plasmid. The *P. acaciae* killer system does not appear to be identical to that of the *K. lactis* killer system however. The *P. acaciae* toxin exhibits both a more limited range of susceptible strains and the toxin itself is distinct from that produced by *K. lactis*. Furthermore, the plasmid sizes vary between the two strains. We are currently examining p*Pac*l-1 and p*Pac*l-2 for homology to the *K. lactis* killer toxin plasmids.

The precise nature and mode of action of the *P. acaciae* toxin are presently unknown. Preliminary experiments suggest that the toxin is proteinaceous. The toxin is heat labile (65 °C, 10 min) and sensitive to proteinase K (P. L. Worsham and P. L. Bolen, unpublished experiments).

Additional studies should reveal whether this toxin might prove useful in biotyping of pathogenic fungi (Polonelli et al. 1983; Morace et al. 1984), as has been demonstrated for other *Pichia* toxins.

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References

Gunge N (1986) Yeast 2:153–162
Gunge N, Tamaru A, Ozawa F, Sakaguchi K (1981) J Bacteriol 145:382–390
Kitada K, Hishinuma F (1987) Mol Gen Genet 206:377–381
Lehmann PF, Lemon MB, Ferencak WJ (1987) Mycologia 79:790–794
Ligon JM, Bolen PL, Hill DS, Bothast RJ, Kurtzman CP (1989) Plasmid 21:185–194
Maniatis T, Fritsch F, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
Meinhardt F, Kempken F, Esser K (1986) Curr Genet 11:243–246
Morace G, Archibusacci C, Sestito M, Polonelli L (1984) Mycopathologia 84:81–85
Polonelli L, Archibusacci C, Sestito M, Morace G (1983) J Clin Microbiol 17:774–780
Radler F, Pfeiffer P, Dennert M (1985) FEBS Lett 29:269–272
Sawant AD, Abdelal AT, Ahearn DG (1988) Appl Environ Microbiol 54:1099–1103
Shepherd HS, Ligon JM, Bolen PL, Kurtzman CP (1987) Curr Genet 12:297–304
Stam JC, Kwakman J, Meijer M, Stuitje AR (1986) Nucleic Acids Res 14:6871–6884
Starmer WT, Ganter PF, Aberdeen V, Lachance M, Phaff HJ (1987) Can J Microbiol 33:783–796
Tokunaga M, Wada N, Hishinuma F (1987) Nucleic Acids Res 15:1031–1046
Wickner RB (1976) Bacteriol Rev 40:757–773
Worsham PL, Goldman WE (1988) Mol Gen Genet
Zorg J, Kilian S, Radler F (1988) Arch Microbiol 149:261–267

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