Pergamon

S0031-9422(96)00037-4

Phytochemistry, Vol. 42, No. 3, pp. 637–639, 1996

Printed in Great Britain
0031-9422/96 $15.00 + 0.00

NONSTEROL RELATED RESISTANCE IN *USTILAGO MAYDIS* TO THE POLYENE ANTIFUNGALS, AMPHOTERICIN B AND NYSTATIN

TIMOTHY JOSEPH-HORNE, NIGEL MANNING,* DEREK HOLOMAN† and STEVEN KELLY‡

Krebs Institute for Biomolecular Research, Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield S10 2UH, U.K.; *Neonatal Screening Laboratory, Sheffield Children's Hospital, Western Bank, Sheffield S10 2UH, U.K.; †Department of Agricultural Sciences, University of Bristol, Institute of Arable Crops Research, Long Ashton Research Station, Long Ashton, Bristol, U.K.

(*Received* 25 *September* 1995)

Key Word Index—*Ustilago maydis*; polyene; antifungal resistance.

Abstract—Two amphotericin B resistant mutants of *Ustilago maydis* were isolated following direct selection from a wild-type population. Each mutant was demonstrated to be cross-resistant to nystatin yet remained sensitive to azoles. Sterol analysis indicated a sterol profile similar to the parent strain, precluding the involvement of an alteration in ergosterol biosynthesis as the cause of polyene resistance.

INTRODUCTION

Published reports on the mechanisms of polyene resistance in fungi predominantly implicate the involvement of lesions in ergosterol biosynthesis [1–4] as the cause of such resistance. Polyenes bind to ergosterol molecules within the phospholipid bilayer, disrupting the membrane resulting in a fungicidal effect [5]. Lesions in enzymes involved in sterol biosynthesis which result in the formation of functional sterols other than ergosterol, and which give rise to polyene resistance, include defects in $\Delta^8 \rightarrow \Delta^7$-isomerase [1, 2] and $\Delta^{5(6)}$-desaturase [2, 3]. Such strains exhibiting these phenotypes accumulate Δ^8-sterols (ergosta-8-en-3β-ol, ergosta-8,22-dien-3β-ol) and Δ^7-sterols (ergosta-7-en-3β-ol and ergosta-7,22-dien-3β-ol), respectively. A lesion in the latter also provides cross-resistance to azole antifungals [6]. Lesions in the P450 14α-demethylase in *Saccharomyces cerevisiae*, if coupled to a second suppressor mutation in the $\Delta^{5(6)}$-desaturase accumulate 14α-methylfecosterol and have also been demonstrated to be polyene and azole cross-resistant [4] with the latter due to the mutation in $\Delta^{5(6)}$-desaturase [7].

Amphotericin B binds to ergosterol in the membranes of cells and this has been demonstrated to be a prerequisite for its fungicidal effect [5]. However, the actual mechanism responsible for cell death remains unclear. Studies conducted by Sokol-Anderson *et al.* [8] indicated that amphotericin B auto-oxidizes, which may involve oxidative damage in the process of cell death following treatment. Studies conducted with *Candida albicans* have shown that resistance to the lethal and lytic effects of amphotericin B may result from growth on media which induce high levels of endogenous catalase [8].

In this paper we report the isolation of two amphotericin B and nystatin cross-resistant mutants of *U. maydis*, which retain a sterol profile similar to the parent strain, ATCC 14826. These mutants were demonstrated to be azole sensitive.

RESULTS

Screening of a wild-type population of *U. maydis* strain ATCC 14826 at the MIC (minimum inhibitory concentration) for amphotericin B (1.1 μM) yielded two isolates, termed N1 and N7. They exhibited resistance to amphotericin B to levels of five- and seven-fold in respect to the parent strain and were demonstrated to be cross-resistant to nystatin (approximately three-fold for each isolate) yet retained parental levels of sensitivity to the azole triadimenol (Table 1).

The sterol profiles were determined in untreated cultures for ATCC 14826, N1 and N7 (Table 2). ATCC

Table 1. Minimum inhibitory concentrations for the polyene antibiotics, amphotericin B and nystatin, and for the azole antifungal, triadimenol

Strain	Polyene antibiotic: Amphotericin B(μM)	Polyene antibiotic: Nystatin (U l^{-1})	Azole triadimenol (μM)
ATCC 14826	1.1	12	1.0
N1	5.5	30	1.0
N7	7.8	35	1.0

‡Author to whom correspondence should be addressed.

Table 2. Sterol profiles for the parent strain ATCC 14826, and the polyene-resistant isolates, N1 and N7

Sterol	Relative sterol content (%)		
	ATCC 14826	N1	N7
Ergosta-tetraenol	2.3	13.1	7.4
Ergosta-5,8,22-trien-3β-ol	–	–	Trace
Ergosterol	55.6	30.9	41.8
Ergosta-7,22-dien-3β-ol	4.2	4.2	1.5
14α-methylfecosterol	–	6.0	4.3
Ergosta-5,7-dien-3β-ol	28.2	11.0	17.8
Ergosta-7-en-3β-ol	2.3	9.8	8.0
4-Methylfecosterol	–	9.6	8.8
Eburicol	1.9	1.9	1.0
4-Methylergosta-7-en-3β-ol	–	4.5	5.0
4,14α-Methylepisterol	–	5.6	5.0
14α-methylergosta-8-en-3,6-diol	–	0.8	Trace
Unidentified sterols	5.5	2.6	0.0

14826 displayed a profile consistent with that published elsewhere (for example see ref. [3]) with the predominant sterol being ergosterol (55.6% of total sterols) and high concentrations of the intermediate ergosta-5,7-dien-3β-ol (28.2% of total sterol). The predominant sterol of isolates N1 and N7 was ergosterol, which accumulated to 30.9% and 41.8% of total sterols, respectively, while the levels of ergosta-5,7-dien-3β-ol were 11.0% and 17.8% of total sterol, respectively. Both isolates accumulated slightly elevated levels of other intermediates compared with their parent strain.

DISCUSSION

Polyene antifungal resistance has previously been attributed to lesions in the ergosterol biosynthetic pathway, such as in the accumulation of Δ^8-desmethyl sterols [1] or Δ^7-desmethyl sterols [2, 3], or in the accumulation of 14α-methylfecosterol [4]. Resistance has also been demonstrated to be conferred through the induction of endogenous catalase in *C. albicans* [8]. The amphotericin B resistant isolates described in this study were shown to be cross-resistant to nystatin but sensitive to the triazole, triadimenol. These mutants readily accumulated ergosterol and other Δ^5-sterols, such as ergosta-5,7-dien-3β-ol while levels of Δ^8-, Δ^7- and 14α-methyl sterols were low and were consistent with a 'parental-like' sterol biosynthesis.

The involvement of amphotericin B resistance through *n*-alkane induction of catalase [8] was not considered to be the mechanism of amphotericin B resistance in mutants N1 and N7, as these were maintained on liquid YEPD media which contains glucose as the carbon source. Other possible mechanisms giving rise to the observed resistance may involve the prevention of antifungal reaching the target molecule, but the examination of this was precluded by the non-availability of labelled amphotericin B.

Mutants N1 and N7, while containing significant levels of ergosterol, 30.9% and 41.8% of total sterol, respectively, were shown to accumulate higher levels of intermediates in comparison to ATCC 14826. The presence of 4-methyl sterols (4-methyl fecosterol, 4-methyl ergosta-7-en-3β-ol and 4,14α-dimethylepisterol (19.7% and 18.8% of total sterols, respectively, compared with 0.0% detected in ATCC 14826) may indicate a reduced ability to demethylate the 4β-methyl in these mutants. However, the levels of ergosterol within these strains was considered sufficient for normal cellular growth and development, as both the growth rate and morphology was similar to the parent strain (unpublished observations).

Overall, the isolation of these mutants represents the first biochemical examination of polyene resistance in a phytopathogen which appears unrelated to an alteration in sterol biosynthesis. The mutation frequency of 10^{-8} implies a single mutation giving rise to the observed resistance, although further work is required to characterize the mechanism. The observed levels of amphotericin B and nystatin resistance, of five- and three-fold, respectively, for N1 and seven- and three-fold, respectively, for N7 are comparable and may indicate a similar mechanism in both strains affording the observed resistance.

The mechanisms of action for polyene antifungals still remains unclear. This study indicates that further examination, involving molecular studies on the basis of resistance, may reveal the mode complete of action of polyene antifungals.

EXPERIMENTAL

Culture conditions. Ustilago maydis strain ATCC 14826 and isolates N1 and N7 were grown on 2% glucose, 2% Difco peptone, 1% Difco yeast extract as liquid medium (YEPD medium) at 25°. Cultures were shaken at 150 rpm. MIC were determined for cells harvested in the late log phase by centrifugation at 4000 rpm and inoculation into 2 ml of YEPD liquid medium to a final concentration of 1×10^{-5} cells ml^{-1}. Polyene antifungals used were amphotericin B (Sigma) and nystatin (Sigma). Stock solns of amphotericin B

were prepd to 1.08×10^{-3} M in the DMSO and nystatin was prepared as a stock 1000 U l^{-1} in 1 ml DMSO. The azole antifungal triadimenol (Bayer) was prepared as a stock 1×10^{-2} M in DMSO. MICs were determined from triplicate treatments with amphotericin B doses between 1.08×10^{-8} M and 1.08×10^{-4} M, nystatin doses between 0.1 U l^{-1} and 100 U l^{-1} and triadimenol doses between 5×10^{-8} M and 1×10^{-4} M. Test cultures used were incubated for 72 hr at 25° and shaken at 150 rpm, with growth assessed by cell counts (haemocytometer) and by determining colony-forming units per milliliter (cfu ml^{-1}).

Mutant isolation. A total of 10^6 sporidia were inoculated onto 20 ml solid YEPD medium + 2% Difco agar containing amphotericin B at a concentration of 5- and 10-fold above ATCC 14826 MIC. The plates were incubated for 5 days at 25°.

Sterol isolation and analyses. Growth of ATCC 14826, N1 and N7 was achieved through incubation at 25° and 150 rpm for 24 hr. Extraction of nonsaponifiable sterols followed the method outlined in ref. [9] with cells saponified at 90° for 1 hr with 3 ml MeOH, 2 ml 60% KOH and 2 ml 0.5% pyrogallol in MeOH. Extraction was completed with 3×5 ml hexane extractions and evapd to dryness under N_2.

Following silylation of samples with BSTFA (20 μl) in toluene (100 μl) for 1 hr at 60°, sterols were analysed by GC using on-column injection, onto a SGE OV1-BP capillary column (25 m × 0.32 mm) at 80°. Following sample injection (1 μl), the oven temp. was increased from 80° to 260° at 40° min^{-1}, before being programmed at 3° min^{-1} to 290°. Carrier gas was H_2 at an inlet pressure of 0.3 kg cm^{-2}. Peaks were determined by flame ionization detection (FID), and quantified using a VG Multichrom data system.

For GC-MS derivatized samples (0.5 μl) were injected using a split/splitless injector (270°) onto an Alltech SE-52 bonded-phase fused silica capillary column (25 m × 0.32 mm). The carrier gas was He (inlet pressure 0.4 kg cm^{-2}), and the temp. programme was 180–280° at 5° min^{-1}. EI mass spectra were obtained at 70 eV.

Peak analysis and identification were realized through reference to current literature [10–12] for relative retention time (RR_t) values and fragmentation ion patterns. Representative data from triplicate analyses are shown.

Acknowledgement—One of our group (T.J-H.) was in receipt of a BBSRC CASE studentship with Long Ashton Research Station.

REFERENCES

1. James, C. S., Burden, R. S., Loeffler, R. S. T. and Hargreaves, J. A. (1992) *J. Gen. Microbiol.* **138**, 1437.
2. Kelly, S. L., Lamb, D. C., Taylor, M., Corran, A. J., Baldwin, B. C. and Powderly, W. G. (1994) *FEMS Letters* **122**, 39.
3. Joseph-Horne, T., Manning, N., Hollomon, D. and Kelly, S. L. (1995) *FEMS Letters* **127**, 29.
4. Taylor, F. R., Rodreguez, R. J. and Parks, L. W. (1983) *J. Bacteriol.* **155**, 64.
5. Kerridge, D. (1986) *Adv. Microbiol. Physiol.* **27**, 1–72.
6. Watson, P. F., Rose, M. E., Ellis, S. W., England, H. and Kelly, S. L. (1989) *Biochem. Biophys. Res. Commun.* **164**, 1170.
7. Kelly, S. L., Lamb, D. C., Corran, A. J., Baldwin, B. C. and Kelly, D. E. (1995) *Biochem. Biophys. Res. Commun.* **207**, 910.
8. Sokol-Anderson, M. L., Brajtburg, J. and Medoff, G. (1986) *J. Inf. Dis.* **154**, 76.
9. Woods, R. A. (1971) *J. Bacteriol.* **108**, 69.
10. Loeffler, R. S. T. and Hayes, A. L. (1990) *Phytochem.* **29**, 3423–3425.
11. Loeffler, R. S. T. and Hayes, A. L. (1992) *Pestic. Sci.* **35**, 7–17.
12. Quail, M. A., Arnoldi, A., Moore, D. J., Goosey, M. W. and Kelly, S. L. (1993) *Phytochemistry* **32**, 273.