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Differential sensitivity of Ustilago maydis to fungal antibiotics on simple and complex media

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Abstract

We have observed that the basidiomycete *Ustilago maydis* can be partially or completely resistant to antibiotics when grown in defined growth media. In synthetic medium based on the fully defined mixture of simple organic compounds and salts *U. maydis* displays near wild-type growth at concentrations of hygromycin that effectively kill cells in complex nutrient media. The antibiotics geneticin, nourseothricin and phleomycin had similar effects. In contrast, the fungicide carboxin was equally effective in all growth media tested. Our observations could guide selection of growth media for genetic transformation of *Ustilago* and other fungi when sensitivity to common antibiotics is used as a selectable marker.

Differential sensitivity of *Ustilago maydis* to fungal antibiotics on simple and complex media

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We have observed that the basidiomycete *Ustilago maydis* can be partially or completely resistant to antibiotics when grown in defined growth media. In synthetic medium based on the fully defined mixture of simple organic compounds and salts *U. maydis* displays near wild-type growth at concentrations of hygromycin that effectively kill cells in complex nutrient media. The antibiotics geneticin, nourseothricin and phleomycin had similar effects. In contrast, the fungicide carboxin was equally effective in all growth media tested. Our observations could guide selection of growth media for genetic transformation of *Ustilago* and other fungi when sensitivity to common antibiotics is used as a selectable marker.

Introduction

Like many other laboratories we have successfully used the fungicide carboxin as a selectable marker in gene transformation (Brachmann et al., 2004; Brachmann et al., 2001; Fernandez-Alvarez et al., 2009; Kojic and Holloman, 2000; Topp et al., 2002) to knock-out variant-specifically histone H3 isotype loci (Verma et al., 2011). After transformation, transformants were cultured under continued selection until stable, non-heterokaryon clones could be isolated (Verma et al., 2011). These cultures were maintained on synthetic minimal media consisting of yeast nitrogen base (YNB), a standard mixture of small organic compounds and salts, with glucose (SD). When attempting to use other standard antibiotic selectable markers such as hygromycin, geneticin, nourseothricin and phleomycin (Brachmann et al., 2004; Kamper, 2004; Kojic and Holloman, 2000), selection was not observed. Other laboratories have used these markers extensively in generating genetic transformants of *Ustilago*, but they used chemically complex media, primarily based on mixtures of yeast extract and bactopeptone supplemented with glucose (YPD) or sucrose (YPS) (Berndt et al., 2010; Brachmann et al., 2004; Brachmann et al., 2001; Lovely et al., 2011; Tsukuda et al., 1988), but also Complete Medium (Garcia-Pedrajas et al., 2008; Holliday, 1974; Lee et al., 1999) or Potato Dextrose (Brachmann et al., 2004; Heidenreich et al., 2008; Zameitat et al., 2007). In our hands too, these complex media, in particular YPS, can be used effectively for antibiotic selection. However, we report here that in simple SD medium aminoglycoside and glycopeptide antibiotics, all larger and more hydrophilic than carboxin, are largely or completely ineffective. We speculate that uptake of these antibiotics depends on *Ustilago* cell membrane transport mechanisms that are absent in the simple SD medium. This observation poses an impediment if one wants to create auxotrophic mutants, e.g. for essential amino acids like leucine (Fotheringham and Holloman, 1990) or nucleotide bases like adenine (Verma et al., 2016) in YNB-based synthetic minimal media.

Materials and Methods

Simple, synthetic-defined media SD and SS consisted of 6.7 g yeast nitrogen base (YNB) without amino acids (Fisher Scientific, BD Difco) with 20 g dextrose (glucose) or sucrose (Sigma-Aldrich), respectively, in 1 L water. pH-buffered SD medium (SDS) was prepared by including 50 mM succinic acid and

adjustment of the pH to 7 with NaOH. Semi-defined, complex growth media YPD and YPS2 contained 10 g yeast extract (BD Difco), 20 g bactopeptone (BD Difco) with 20 g glucose or sucrose, respectively, in 1 L water. In addition to this rich complex medium (Tsukuda et al., 1988), we also tested the poorer formulation YPS1 with only 4 g yeast extract and 4 g bactopeptone per L (Brachmann et al., 2004). *Ustilago* growth rates and antibiotic effects were indistinguishable between YPS1 and YPS2. For plate cultures, media were supplemented with 15 g agar (Fisher Scientific) per L. Unless specified, media were autoclaved without adjusting the pH of approximately 6.5.

Aliquots of stock solutions of antibiotics were added to the desired final concentrations into autoclaved simple or complex agar media, after cooling to approximately 55 C. Filter-sterilized hygromycin (Sigma-Aldrich) and geneticin (G418, Gibco BRL) stocks were 50 mg/mL water; nourseothricin (NTC, Jena Bioscience, Germany) was 100 mg/mL water; phleomycin (InVivogen) was 20 mg/mL stock as purchased. Carboxin (5,6-dihydro-2-methyl-1,4-oxathi-ine-3-carboxanilide; Vitavax) was 10 mg/mL methanol, diluted from a 34% suspension in methanol, a kind gift from S. Gold (Athens, GA) and used at 3 µg/mL (Verma et al., 2011).

Wildtype *U. maydis* 521 haploid strain FGSC 9021 (Verma et al, 2011) was revived from –70 C storage in 50% glycerol, grown on SD plates at 30 C and single colonies were grown overnight in 50 mL SD or YPD at 30 C at 150 rpm in 125 mL flasks. Multiple samples of 250 cells in 10 microliter medium, after volume adjustment based on hematocytometer counting, were spotted on 100 mm diameter Petri dishes with 40 ml agar medium, allowed to dry, and incubated in the dark at 30 C for at least 1 week. Note that initial inoculum sizes increased as colonies grew, spreading across the agar surface (Fig. 1).

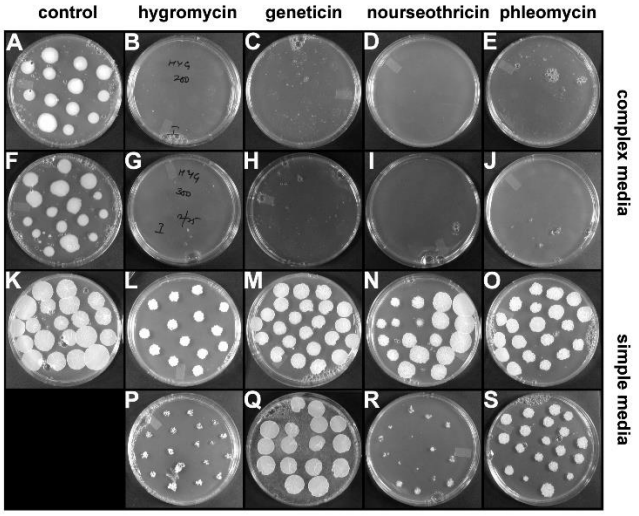

Figure 1. Examples of *U. maydis* growth on solid media. Multiple aliquots of 10 µL of *Ustilago* suspension culture containing 250 cells were spotted on agar plates with complex or simple nutrient media and with sucrose or glucose (see Legend of Table 1) and grown in the dark at 30 C for 3 (A, F) or 6 days (B-E, G-S). A-J: complex media; K-S: simple media. Table 1 scores are indicated between square brackets. **A.** control, YPS1 [+++]; **B.** hygromycin, 200 µg/mL, YPS1 [NO]; **C.** geneticin, 200 µg/mL, YPS1 [NO]; **D.** nourseothricin, 150 µg/mL, YPS1 [NO]; **E.** phleomycin, 50 µg/mL, YPS1 [NO]; **F.** control, YPS2 [+++]; **G.** hygromycin, 300 µg/mL, YPS1 [NO]; **H.** geneticin, 250 µg/mL, YPS2 [NO]; **I.** nourseothricin, 300 µg/mL, YPS2 [NO]; **J.** phleomycin, 20 µg/mL, YPS2 [NO]; **K.** control, SD [+++]; **L.** hygromycin, 150 µg/mL, SD [++]; **M.** geneticin, 100 µg/mL, SD [+++]; **N.** nourseothricin, 150 µg/mL, SD [++]; **O.** phleomycin, 15 µg/mL, SD [+++]; **P.** hygromycin, 250 µg/mL, SD [+]; **Q.** geneticin, 300 µg/mL, SD [+++]; **R.** nourseothricin, 300 µg/mL, SD [+]; **S.** phleomycin, 50 µg/mL, SD [++]. Note the somewhat variable size of the 10 µL spots applied on plates like A where large colonies grow in only 3 days and plates like B, where the application spots remain faintly visible but microscopic colonies are never detected.

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Results and Discussion

In our research to create selective knock-outs for the two single locus histone H3 variants in *Ustilago*, successful transformation at single loci was achieved using the selectable marker carboxin on simple defined (SD) growth media based on yeast nitrogen base (YNB) and glucose (Verma et al., 2011). The mutant succinate dehydrogenase cassette (Brachmann et al., 2004) which confers resistance to carboxin (Keon et al., 1991) was stably integrated in the *Ustilago* genome at the knock-out locus. To study the single remaining histone H3 variant function, we wished to replace its promoter by that of the inactivated gene, switching between the cell-cycle regulated H3 promoter and the constitutive H3 promoter (Verma et al., 2011).
In the search for a second usable selectable marker, we were surprised by abundant growth on SD plates with hygromycin, geneticin (G418), nourseothricin (NTC) and phleomycin (Fig. 1) at antibiotic concentrations that were described as effective by other laboratories using *Ustilago* (Berndt et al., 2010; Brachmann et al., 2004; Brachmann et al., 2001; Garcia-Pedrajas et al., 2008; Heidenreich et al., 2008; Kamper, 2004; Kojic and Holloman, 2000; Lee et al., 1999; Lovely et al., 2011; Tsukuda et al., 1988; Zameitat et al., 2007). Reviewing growth media used by these laboratories, none had used simple media like our YNB-based one with glucose. We confirmed that indeed these antibiotics were effective against wild type *Ustilago* strains when yeast extract- and bactopeptone-based media were used (Fig. 1) at the effective concentrations described (Table 1).

We explored some of the possible factors involved to find conditions that would allow use of these antibiotics as selectable markers in simple media during the development of auxotrophic markers, such as the *ade2* knockout (Verma et al., 2016). The effects of the composition of growth media during genetic transformation and selection of *Ustilago* has been recognized by others. For instance, the inclusion of high concentrations of sorbitol in protoplast transformation of *Ustilago* is required for the stabilization of the protoplasts but it reduces the effectiveness of antibiotic selection (Kojic and Holloman, 2000).

Table 1. *U. maydis* growth on solid media.

	Medium components (g/L)					
Medium	yeast extract	bacto-peptone	YNB	sucrose	glucose	succinic acid
YPS1	4	4		20		
YPS2	10	20		20		
YPD	10	20			20	
SS			6.7	20		
SD			6.7		20	
SDS			6.7		20	5.9

Table 1, *U. maydis* growth on solid media, continued.

	Antibiotics (µg/mL)										
Medium	Control	Hygromycin			Geneticin	Nourseothricin			Phleomycin		Carboxin
	0	150	200-250	300	100-300	100	150	250-300	10-20	30-50	3
YPS1	+++		NO	NO	NO	NO	NO	NO	NO	NO	
YPS2	+++		NO	NO	NO	NO	NO	NO	NO	NO	NO
YPD	+++	NO	NO			NO	NO			NO	
SS	+++	++	+			+	(+)			+	NO
SD	+++	++	+	(+)	+++	++	++	+	+++	++	NO
SDS	+++	+++	++			NO	NO			(+)	

Complex agar media (YPS1, YPS2, YPD) with yeast extract and bactopeptone and simple agar media (SS, SD, SDS) with yeast nitrogen base containing sucrose, glucose and/or succinate buffer (see Materials and Methods). Growth at 30 C in dark. Examples of scored plates are shown in Fig. 1. Scores: +++ : sizable colonies by day 3 as in untreated controls; ++ : visible colonies by day 3; + : small macroscopic colonies by day 6 (+) : microscopic colonies by day 6; NO : no microscopic colonies after 6 or more days. Empty table fields represent combinations of medium and antibiotic that were not tested.

The uptake of sugars by fungi is generally mediated by sugar transporters (Goncalves et al., 2016) and specific sucrose (MW 342 D) transporters have been identified in *Ustilago* (Wahl et al., 2010). That sucrose transporters may contribute to the entry of the only slightly larger antibiotics is suggested by lesser growth when antibotics are present in simple medium with sucrose (SS) than with glucose (SD) (Table 1). The aminoglycosides hygromycin (528 D), geneticin (497 D) and nourseothricin (multiple components between 499 and 883 D) are only marginally larger than sucrose, although the glycopeptide phleomycin is significantly larger (1428 D). Note that all these antibiotics contain amines that would create increasingly more positively charged antibiotics as cells grow and the unbuffered media acidify. That the charged state may impede cell entry is suggested by the observation that nourseothricin and phleomycin in pH-buffered medium (SDS) are more effective (Table 1). The smaller molecular weight (235 D) but especially the hydrophobic character of carboxin is probably responsible for its effective uptake and action in both simple and complex media (Table 1).

The major difference observed in this study is the limited antibiotic effectiveness in the simple media in which the organic components all have molecular weights less than 450 D, and the complex media with effective antibiotic action and nutrient molecules in yeast extract and bactopeptone with molecular weights that are much larger (Fig. 1; Table 1). The requirement of nutrient uptake from these media will certainly activate cellular import mechanisms, transmembrane transporters or endocytosis, that will also facilitate the effective uptake of antibiotics into the cells.

Conclusions

Using aminoglycoside and glycopeptide antibiotics as selectable markers in gene transformation of fungi like the basidiomycete *Ustilago maydis*, one must employ growth media that will induce uptake of

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complex nutrients to assure concomitant uptake of antibiotics to confer effective selection. In minimal media, *Ustilago* cells exclude aminoglycoside antibiotics to such a degree that effective selection of antibiotic-resistant transformants cannot be achieved.

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