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Introduction and maintenance of prokaryotic DNA in *Ustilago violacea*

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SUMMARY

A strain of the basidiomycete, *Ustilago violacea*, was transformed with a prokaryotic plasmid, pMP4-1, which confers resistance to neomycin. *U. violacea* transformants were selected at a frequency of 5 per μg pMP4-1 DNA. Such transformants were at least 8-fold more resistant to neomycin than was the untransformed recipient *U. violacea*. Enzyme activity associated with the neomycin resistance gene was also found in the transformants. Southern DNA–DNA hybridization detected pMP4-1-derived sequences in both nuclear and mitochondrially-associated DNAs from transformants. The patterns of hybridization suggested integration of pMP4-1 sequences into the respective genomes. DNA from the nuclear fraction of *U. violacea* transformants failed to produce *E. coli* transformants resistant to neomycin or to carbenicillin. In contrast, DNA from the mitochondrially-associated fraction in *U. violacea* transformants produced *E. coli* transformants resistant to neomycin. The *E. coli* transformants contained a pMP4-1-derivative, pWP8, which was subsequently shown by Southern blot analysis to harbor *U. violacea* mitochondrial DNA. Thus, a prokaryotic plasmid can be used to transform the eukaryote *U. violacea* and acquire endogenous sequences from this organism.

INTRODUCTION

Although saprophytic and phytopathogenic basidiomycetes have been used by both geneticists and phytopathologists for genetic studies, limited linkage data are available. *Ustilago* species are among the few fungal pathogens that are as technically suited for genetic analysis as *Saccharomyces, Neurospora*, and *Aspergillus*. Much work has focused on *U. maydis* due to its economic importance as a pathogen of corn [1]. *U. violacea*, a pathogen of

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members of the Caryophyllaceae, has also been well studied [2]. The genetics of phytopathogenicity is now 'ripe for analysis' in this species [2] and would be facilitated by molecular biological approaches. The development of transformation system for *U. violacea* is a first step in this direction [3].

In this report we describe the use of a prokaryotic plasmid bearing a neomycin resistance gene to recover endogenous sequences from *U. violacea* which could promote extrachromosomal DNA replication in this organism. Concomitant introduction of a prokaryotic plasmid and selection of the resulting neomycin-resistant fungal cells are demonstrated. In addition, mitochondrial sequences of *U. violacea* are recovered using this system.

MATERIALS AND METHODS

Cultures and plasmids

E. coli L-O [4] was the antibiotic-susceptible recipient for all bacterial transformations. *U. violacea* 2.C436*w* [5; obtained from E.D. Garber, Univ. of Chicago] was the white haploid strain used as recipient for fungal transformations. Plasmid pMP4-1 confers resistance to carbenicillin and to aminoglycoside antibiotics neomycin and G418 by virtue of the gene for aminoglycoside 3′-phosphotransferase II (APH(3′)-II) derived from Tn*5*. The pMP4-1 plasmid was produced by cloning the APH(3′)-II gene as a *Bam*HI-*Hin*dIII fragment into similarly-digested pBR322 [4].

Enzymes and chemicals

β-glucuronidase (Type HA4) from *Helix aspersa* and lyticase (from *Arthrobacter luteus*) were purchased from Sigma Chemical Co., St. Louis, MO. Nick translation kit (DNase I + DNA polymerase I) and restriction endonucleases were obtained from International Biotechnologies, Inc., New Haven, CT. Carbenicillin sulfate and neomycin sulfate were purchased from Sigma Chemical Co., St. Louis, MO. Nitrocefin was a generous gift of S. Lerner. Labelled nucleotides, α-[^{32}P]-dATP (>600 Ci/mmol) and γ-[^{32}P]-ATP (1000–3000 Ci/mmol) were obtained from New England Nuclear, Boston, MA.

Preparation of sonic extracts and enzyme assays

Extracts from strains were prepared by sonication as previously described [6]. β-lactamase activity in crude cellular extracts was assayed qualitatively by the production of orange color by cleavage of the chromogenic cephalosporin, nitrocefin [7]. APH (3′)-II activity in crude extracts was measured by the radioenzymatic phosphocellulose filter binding assay [8]. Alternatively, crude extracts were separated by non-denaturing polyacrylamide gel electrophoresis (PAGE). Separated samples were then assayed directly in the gel according to the procedure of Sprengel et al. [7].

Measurement of antibiotic susceptibilities

Minimal Inhibitory Concentrations (MICs) of antibiotics for strains were determined using a two-fold serial dilution method [8] on agar plates.

Transformation protocols

The transformation of susceptible *E. coli* was by the $CaCl_2$ method described by Maniatis et al. [9]. *U. violacea* protoplasts were transformed as described by Wang et al. [1], except that β-glucuronidase (50 000 U) and lyticase (1% final concentration) replaced Novozym 234 in the production of protoplasts. Protoplasts of *U. violacea* 2.C436*w* were incubated with 5 μg of pMP4-1 DNA and were treated with $CaCl_2$ and polyethylene glycol. Five separate transformations were performed. Cells from each of these transformations (10^6 protoplasts and 5 μg DNA) were spread onto regeneration medium [1] containing 10 mg neomycin per ml and were incubated at 25°C for 12 days.

Mitotic stability of putative transformants

The mitotic stability of putative transformants was estimated by inoculating single colonies of isolates maintained on selective media into broth with or without selective pressure (antibiotics) and allowing growth to log phase ($A_{600} = 1$). At this point cultures were diluted 200-fold again into broth either with or without antibiotic and allowed to grow to log phase. Cells were passaged at least 3 times in this manner. After each growth phase to $A_{600} = 1$, aliquots were diluted and spread onto selective and

non-selective media and the proportion of colonies retaining the drug-resistant phenotype were determined. The fraction of cells losing the transformed phenotype per generation was estimated by the following formula:

$$\mu = 1 - 10^{\frac{\log P_g}{g}}$$

where μ is the loss per generation and P_g is the observed proportion of antibiotic-resistant colonies (compared to total) after g generations [10]. This method of estimation was found to yield numbers comparable to those obtained using either the method of Murray and Szoskak [11] or that of Dani and Zakian [12].

Isolation and purification of DNAs from U. violacea

Nuclei and mitochondria from *U. violacea* were purified by differential lysis and centrifugation according to the procedure of Wills et al. [13]. Total DNA or DNA isolated from purified nuclei or purified mitochondria was extracted according to the methods of Cryer et al. [14]. The DNAs were further purified in cesium chloride (CsCl) bisbenzamide buoyant density gradients [15].

Characterization of DNAs

Methods for digestion with restriction endonucleases were as described by the manufacturer. Electrophoresis was performed in 0.8% agarose at 1.3 V/cm^3 for 6–8 h. The buffer system contained 89 mM Tris-hydrochloride, 8.9 mM boric acid, and 2.5 mM EDTA (pH 8.3). Nick translation of DNAs with $\alpha[^{32}P]$-dATP for use as probes, transfer of DNAs to nitrocellulose, DNA–DNA hybridization, and autoradiography were according to the methods of Maniatis et al. [9]. A purified 1.5-kb *Eco*RI fragment from nuclear DNA of untransformed *U. violacea* 2.C436*w* was used to probe for cross-contamination of mitochondrial preparations with nuclear DNA sequences.

We wished to investigate whether transforming DNA in the *U. violacea* transformants was integrated or if some of the detected sequences were independent of the nuclear and/or mitochondrial genomes. To do so, DNAs from purified nuclei and mitochondria were prepared from the *U. violacea* transformants [16] and these were then purified in CsCl bisbenzamide gradients [18]. DNA fractions from two of the independent *U. violacea* transformants were used separately for these experiments. Susceptible *E. coli* L-O were transformed independently with the nuclear and mitochondrial DNA fractions.

RESULTS

Transformation of U. violacea

Colonies of *U. violacea* appeared at a frequency of 5 per μg pMP4-1 DNA. Typically, transformation plates contained between 15–30 colonies. In the absence of transforming DNA, protoplasts plated on regeneration medium (containing 10 mg NM per ml) failed to yield colonies up to 18 days at 25°C. This was true even when the number of untransformed renegerable protoplasts spread was as high as 1×10^8 per plate. At 25°C, both transformed and untransformed protoplasts grew on regeneration medium lacking antibiotic within 3–5 days. Similar results were obtained in all 5 separate transformation experiments.

A colony from each of the 5 independent transformations was chosen for further study. All five were at least 8-fold more resistant to neomycin than their progenitor strain, 2.C436*w* (see Table 1). These haploid strains were examined for mitotic stability of this acquired trait. After 29 generations of mitotic division in selective media, the neomycin-resistant phenotype was retained in approx. 100% of cells for each of the five transformants. However, after 14 generations of mitotic growth without selective pressure (see Table 2), 71–80% of cells maintained the neomycin-resistant phenotype. After 24 generations in non-selective media only 3–8% of these cells retained the transformed phenotype.

Characterization of U. violacea transformants

All five independently-isolated *U. violacea* transformants had identical characteristics with regard

Table 1

Antibiiotic susceptibilities and enzyme activities in *U. violacea* and *E. coli* strains

Strain	MICs[a] (μg/ml) of the following drugs[b]:		Enzyme activities[c]	
	NM	CB	APH(3′)-II	β-lactamase
2.C436*w*	1250	N.D.[d]	−	−
2.C436*w*(pWP8)[e]	>10000	N.D.	+	−
L-O(pWP8)	800	800	+	+
L-O(pMP4-1)	800	800	+	+
L-O	3.12	<12.5	−	−

[a] MIC, minimal inhibitory concentration.

[b] NM, neomycin; CB, carbenillcillin.

[c] APH(3′)-II activity in crude sonic extracts determined by radioenzymatic phosphocellulose filter binding assay [8] or by the non-denaturing polyacrylamide gel electrophoresis assay [7]; β-lactamase activity in crude sonic extracts determined qualitatively as production of orange color by the cleavage of the chromogenic cephalosporin, nitrocefin.

[d] N.D., not done since *U. violacea* are not sensitive to this drug.

[e] Represents each of 5 independent *U. violacea* transformants, since the results were identical.

to antibiotic susceptibilities, enzyme activites, and observed DNA content. The MICs of the transformants and the enzyme activities present in crude extracts of these strains are shown in Table 1.

U. violacea strains 2.C436*w* had an MIC of 1250 μg per ml neomycin. The spontaneous mutation rate of this strain for growth on 10 mg neomycin per ml was less than 1×10^{-9}. As previously mentioned, the *U. violacea* transformants were at least 8-fold more resistant to neomycin than 2.C436*w*. All *U. violacea* strains tested grew readily on plates containing 10 mg carbenicillin per ml. The non-denaturing PAGE assay [7] confirmed the presence of APH(3′)-II enzyme activity in the *U. violacea* transformants. No such activity was detected in untransformed *U. violacea* 2.C436*w*.

Fate of transforming DNA in U. violacea transformants

The probes used to characterize the nuclear and mitochondrial DNA fractions of the five independent *U. violacea* transformants were linearized pMP4-1 and the 1.8-kb *Hin*dIII-*Bam*HI fragment from pMP4-1 bearing the APH(3′)-II gene (hereafter referred to as the APH(3′)-II gene probe). Both the nuclear and mitochondrial fractions of all 5

Table 2

Mitotic stability[a] of transformed phenotype in *U. violacea* on non-selective medium

Generation	Trans[b] 1		Trans 2		Trans 3		Trans 4		Trans 5	
	% Cells[c]	μ_B[d]	% Cells	μ_B	% Cells	μ_B	% Cells	μ_B	% Cells	μ_B
14	78	0.018	74	0.022	80	0.016	72	0.024	81	0.015
19	27	0.067	38	0.050	22	0.077	29	0.064	31	0.060
24	3	0.081	4	0.126	5	0.118	3	0.136	8	0.100

[a] Mitotic stability calculations were made with the per cent cells containing plasmid at time 0 normalized to 100%. Cell were grown to log phase and serially passaged up to 24 generations in the specific media.

[b] Trans, transformant.

[c] % Cells, percent cells maintaining transformed phenotype (i.e. neomycin resistance).

[d] μ_B, plasmid loss per cell per generation.

transformants showed hybridization to both of these probes.

The results of hybridization with pMP4-1 are presented in Fig. 1A. The pMP4-1 plasmid hybridized strongly with itself (lane 8). For the nuclear fraction *Bam*HI digestion produced a 9.2-kb band of hybridization (Panel A, lanes 1,2,4,5,7) to linear pMP4-1. The digested DNA from purified mitochondria (lanes 10, 11, 13–15) produced a 7.43-kb band of hybridization to pMP4-1. Thus, sequences from pMP4-1 were present in the transformed *U. violacea.*

In the second set of hybridizations (Panel B), the nuclear and mitochondrial DNAs digested with *Hin*dIII and *Bam*HI allowed detection of an intact APH)3′)-II gene in the transformants. The APH (3′)-II gene probe hybridized to a 1.8-kb fragment in the nuclear DNA fractions from the transformants. Interestingly, in the mitochondrial fractions, the APH(3′)-II gene probe produced the same 7.43-kb band of hybridization which had been detected with the pMP4-1 probe. No cross-contamination with nuclear DNA was detected (Panel A, lane 16). Thus the observed hybridization appears to be exclusively to sequences associated with the mitochondria.

In these experiments, no hybridization was detected for the parental *U. violacea* 2.C436*w* strain (Panel A, lanes 3,6,12; Panel B, lane 4), or for *U. violacea* transformants which had subsequently lost their neomycin-resistant phenotype (not shown). The only hybridization to parental *U. violacea* DNAs was that observed for the nuclear probe to the nuclear DNA fraction from this strain (panel A, lane 17). Comparison of the degree of intensity of hybridization to one copy equivalent of pMP4-1 (Panel A, lane 9) suggests the presence of approximately one copy of these sequences in the nuclear components of three *U. violacea* transformants (lanes 1, 4, and 7) and multiple copies for at least one transformant (lane 5).

Transformation of E. coli with DNA from U. Violacea transformants

When crude DNA from purified mitochondria of *U. violacea* transformants was used to transform bacteria, *E. coli* L-O transformants appeared at a frequency of 12 per μg *U. violacea* DNA on TYE agar [16] plates containing 200 μg carbenicillin or neomycin per ml. Alternatively, when fractions were used from CSCl bisbenzamide gradients on which *U. violacea* transformant DNA had been separated, DNA from the fraction just below the mitochondrial band was found to transform *E. coli* at a frequency of 80 per μg. Despite numerous attempts to produce *E. coli* transformants using the nuclear DNA fraction, no such transformants were observed. The eighteen tested *E. coli* transformants produced from mitochondrially-associated DNA had identical characteristics, including antibiotic susceptibilities, enzyme activities and plasmid DNA content.

E. coli L-O(pWP8) is a representative re-transformant and was resistant to the same high level of carbenicillin and neomycin as L-O transformed with pMP4-1 (see Table 1). In addition, the L-O (pWP8) transformants were all highly resistant to several aminoglycosides which are not normally modified by APH(3′)-II, such as gentamicin and streptomycin (not shown). Crude extracts of these transformants exppressed the β-lactamase activity also present in extracts of *E. coli* L-O(pMP4-1). However, a lower level of APH(3′)-II activity was detected in extracts of *E. coli* L-O-(pWP8) than in those of L-O(pMP4-1).

The plasmid, pWP8, was detected in all bacterial transformants tested. This plasmid is approximately 9.0-kb, compared to the 5.8-kb pMP4-1 plasmid. Plasmid pMP4-1 and pWP8 were compared by restriction endonuclease digestions (Fig. 2) and by DNA–DNA hybridization (not shown) and were thereby shown to have some similarities and some substantial differences between their organizations. In addition to acquisition of new sequences, pWP8 contained new *Bal*I, *Bam*HI, *Sma*I and *Sal*I sites. It had lost *Eco*RI and *Hin*DIII sites. The restriction maps suggested that the carbenicillin resistance gene was intact.

Two fragments (0.77-kb *Pst*I and 1.59-kb *Bam*HI) and portions of the 4.9-kb *Pst*I fragment from pWP8 did not hybridize with either the pBR322-derived portion of pMP4-1 or the fragment bearing

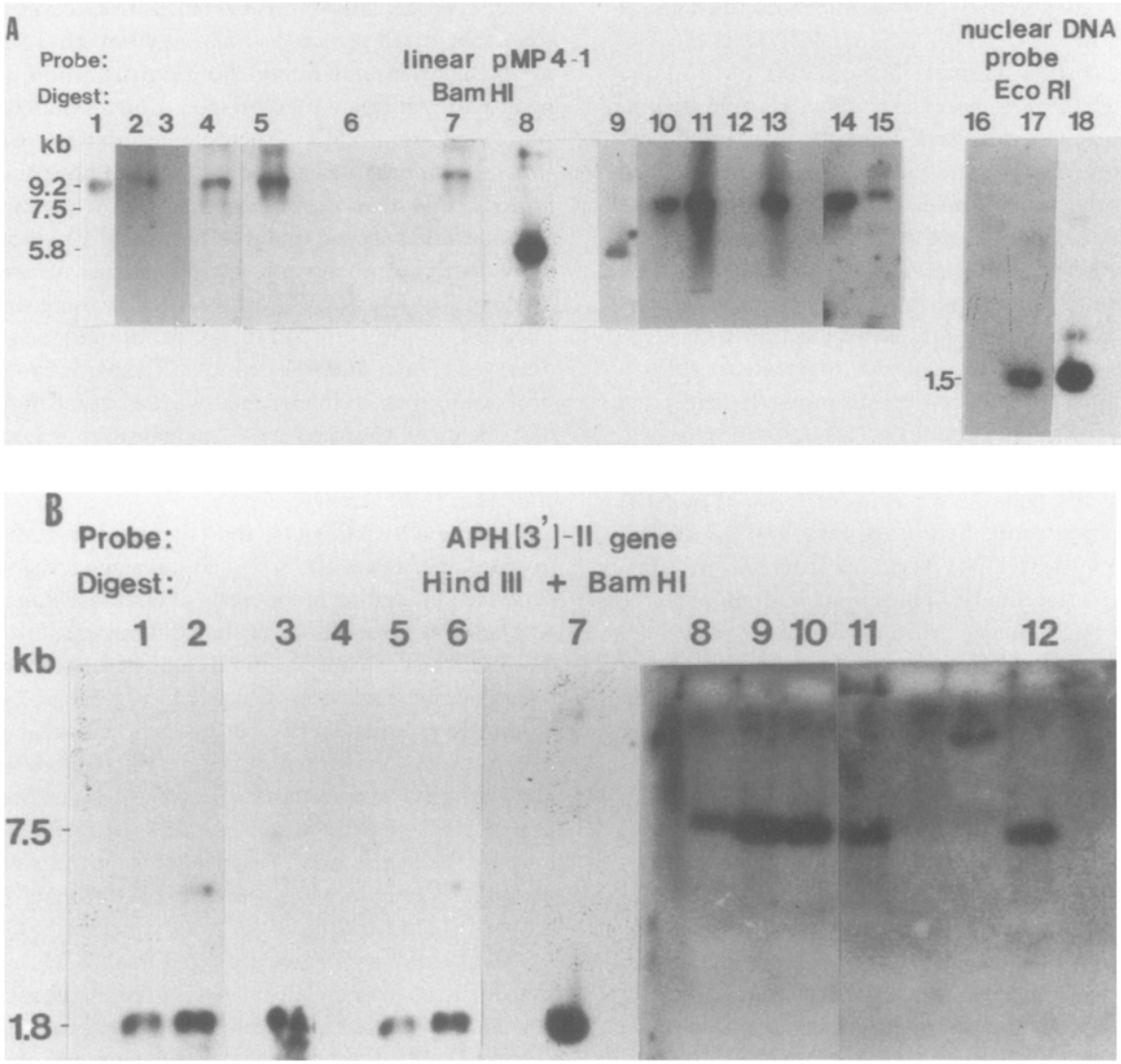

Fig. 1. Southern hybridization of *U. violacea* transformant DNAs. Approximately 3–5 μg *U. violacea* DNA was used in each case. Panel A: Linear *Bam*HI-digested pMP4-1, nick translated with α-[^{32}P]-dATP, was used as the probe for lanes 1–15; a cloned 1.5-kb *U. violacea* nuclear DNA fragment was the probe for lanes 16–18. For lanes 1–15, all DNAs were digested with *Bam*HI before loading onto the gel; DNAs in lanes 16 and 17 were digested with *Eco*RI before electrophoresis. Lanes 1,2,4,5 and 7 contained nuclear DNA from 5 *U. violacea* transformants; lanes 3,6, and 17 were nuclear DNA from untransformed *U. violacea* 2.C 436*w*. Lanes 9–11 and 13–15 contained DNA isolated from purified mitochondria for 5 *U. violacea* transformants; lanes 12 and 16 were mitochondrial DNA from untransformed *U. violacea* 2.C436*w*; lanes 8 and 9 contained 0.5 μg and 1 ng, respectively, of *Bam*HI-digested pMP4-1 DNA; lane 18 contained 0.5 μg of 1.5-kb nuclear DNA fragment from *U. violacea*. Film for lanes 1,4–9, and 18 was exposed at −70°C for 12 h; for lanes 2,3,10–17 exposure was for 28 h at −70°C. Panel B: The 1.8-kb *Hin*dIII-*Bam*HI fragment from pMP4-1 containing the APH(3′)-II gene was used as the radiolabelled probe. DNAs in all lanes were digested with *Hin*dIII and *Bam*HI before electrophoresis. Lanes 1–3,5, and 6 contained nuclear DNA from 5 *U. violacea* transformants; lane 4, nuclear DNA from untransformed *U. violacea* 2.C436*w*; lanes 8–12, mitochondrially-associated DNA from 5 *U. violacea* transformants; lane 7, 0.3 μg *Hin*dIII-*Bam*HI-digested pMP4-1. Film for lanes 1–7 was exposed at −70°C for 12 h; for lanes 8–12 exposure at −70°C was for 32 h.

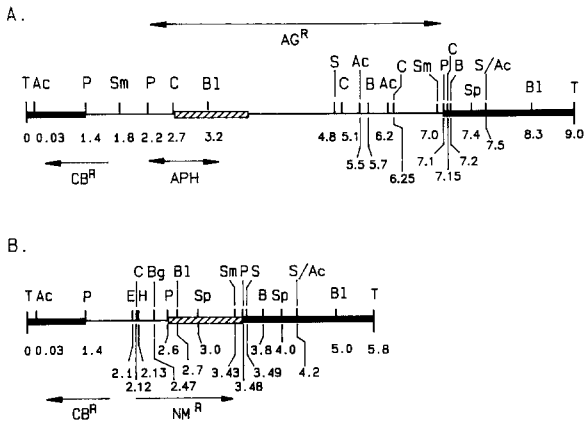

Fig. 2. Restriction maps of (a) pWP8, linearized with *Tth* 111 I, and (b) pMP4-1, linearized with *Tth* 111 I. The intact APH(3′)-II gene and the β-lactamase gene (from pBR322) are indicated by $\overrightarrow{NM^r}$ and $\overleftarrow{CB^r}$, respectively. ▬, indicates material in pWP8 derived directly from pMP4-1 with slight or no alteration, and the corresponding region on pMP4-1; ▨, indicates material in both pWP8 and pMP4-1 which hybridizes with the 0.8-kb *Pst*I fragment containing most of the APH(3′)-II gene; putative locations of the generalized aminoglycoside resistance and what remains of the APH gene in pWP8 are indicated by $\overleftrightarrow{AG^r}$ and $\overleftrightarrow{APH}$, respectively. Sites for cleavage by restriction endonucleases are indicated by T, *Tth* 111 I; Ac, *Acc*I; P, *Pst*I; Sm, *Sma*I; C, *Cla*I; Bl, *Bal*I; S, *Sal*I; B, *Bam*HI; Sp, *Sph*I; E, *Eco*RI; H, *Hin*dIII; Bg, *Bgl*II.

APH(3′)-II gene, suggesting that these additional sequences were acquired from *U. violacea*. The 4.9-kb and 0.77-kb *Pst*I fragments from pWP8 were each used as probes to search for homology with the *U. violacea* genome. The results for the 0.77-kb *Pst*I probe are shown in Fig. 3a. This fragment hybridized with a 0.4-kb fragment from mitochondrial DNA of untransformed *U. violacea* 2.C436*w*. Except for the pWP8 control, hybridization was not detected for any other DNA, including nuclear *U. violacea* DNA. Furthermore, the 4.9-kb *Pst*I fragment probe hybridized with a 2.9-kb fragment in *Pst*I-digested mitochondrial DNA from untransformed *U. violacea* 2.C436*w* (see Fig. 3b).

Experiments using pWP8 to transform *U. violacea* 2.C436*w* to neomycin resistance have yielded transformants at frequencies two-to-three times that seen with pMP4-1 DNA. Preliminary investigations of mitotic stabilities for such transformants suggest that in the absence of selection they lose the transformed phenotype at a rate comparable to that of 2.C436*w* transformed with pMP4-1.

Fig. 3. Southern hybridization of fragments from pWP8 to genomic DNAs from untransformed *U. violacea*. Methods were as described in the text. a: The 0.77-kb *Pst*I fragment from pWP8, nick translated with α-[^{32}P]-dATP, was used as the probe. Lanes A–D were: lane A, pWP8, 1 μg, digested with *Pst*I; lane B, the 0.77-kb *Pst*I fragment of pWP8, 0.3 μg; lane C, mitochondrial DNA, 3 μg, from untransformed *U. violacea* 2.C436*w* digested with *Pst*I; lane D, nuclear DNA, 3 μg, from untransformed *U. violacea* 2.C436*w* digested with *Pst*I. b: The radiolabelled 4.9-kb *Pst*I fragment from pWP8 was used as the probe. Lanes A–E were: lane A, pWP8, 1 μg, digested with *Pst*I; lane B, the 4.9-kb *Pst*I fragment from pWP8, 0.3 μg; lane C, mitochondrial DNA, 3 μg, from untransformed *U. violacea* 2.C436*w* digested with *Pst*I; lane D, nuclear DNA, 3 μg, from untransformed *U. violacea* 2.C436*w*; lane E, pMP4-1, 1.5 μg, digested with *Hin*dIII and *Bam*HI.

DISCUSSION

In order to aid in the molecular characterization of *U. violacea*, bacterial plasmid pMP4-1 was chosen for transformation of this phytopathogen. An aminoglycoside-resistance marker was used previously to transform fungi without further modification of the gene to improve its expression in these organisms [17,18]. Plasmid pMP4-1 was chosen to transform *U. violacea* because it contained a similar aminoglycoside-resistance marker and because as a prokaryotic plasmid it should not replicate in *U. violacea*. Stable transformation would thus require integration of all or part of pMP4-1 into the *U. violacea* genome or acquisition by pMP4-1 of *U. violacea* sequences which allow autonomous replication. Neomycin-resistant *U. violacea* transformants were obtained at approximately 5 per μg pMP4-1 DNA. This frequency was approximately one-third that which we observed previously when protoplasts were transformed with a plasmid bearing a *hygB* (hygromycin-resistance) gene, engineered for eukaryotic expression [3]. In this report, hybridization results suggested integration of pMP4-1 sequences into the nuclear genome, while leaving the APH(3′)-II gene intact. However, DNA preparations associated with the mitochondria from the *U. violacea* transformants described in this report could re-transform susceptible *E. coli* to carbenicillin or to aminoglycoside resistance. The plasmid recovered in this manner, pWP8, is larger than the original plasmid, pMP4-1, as evidenced by restriction enzyme analysis and since confirmed by electron microscopy (A. Bej, unpublished observations). The lower-level APH(3′)-II activity found in *E. coli* bearing pWP8 suggests that in the formation of pWP8 some portion of the APH(3′)-II gene was disrupted (e.g. the loss of *Sph*I and *Sma*I sites and the movement of a *Bal*I site). An alternative explanation is that the larger pWP8 plasmid was found in fewer copies per cell, resulting in reduced APH(3′)-II gene dosage.

We propose that pWP8 was produced by recombination between pMP4-1 and some part of the *U. violacea* mitochondrial genome. This recombination yielded a plasmid ('pWP8') with a rearranged APH(3′)-II and with sequences acquired from *U. violacea* capable of conferring generalized aminoglycoside resistance in bacteria. Aminoglycoside-resistance genes have been described in the mitochondria of yeast [19]. We have demonstrated that both the 0.77-kb and 4.9-kb *Pst*I fragments of pWP8 hybridize to mitochondrial DNA from untransformed *U. violacea* 2.C436*w* (Fig. 3a and b). In addition, the cloned 4.9-kb *Pst*I fragment confers generalized aminoglycoside resistance in *E. coli* (A. Bej and M. Perlin, unplubished observations). The data also suggest that the pWP8 plasmid was associated with the *U. violacea* mitochondria (Fig. 1), which is of interest since the selective agent used for transformation, neomycin, elicits its effect only on the mitochondrial ribosomes in *U. violacea* [20].

The fact that crude extract of putative *U. violacea* 2.C436*w* transformants could transform *E. coli* to carbenicillin resistance could be explained in two ways. Plasmid pWP8 existed as an autonomous molecule in transformed *U. violacea*; this molecule could be a replicon or could be frequently produced by recombination between integrated pMP4-1 sequences and the mitochondrial genome. Or, sequences from pMP4-1 integrated into the *U. violacea* genome; subsequent transformation of *E. coli* with fragmented *U. violacea* chromosome allowed cells to receive a fragment containing some of these sequences which fortuitously recircularized to yield pWP8. The mitotic stability of the transformed phenotype in the *U. violacea* transformants is consistent with both a replicon containing an ARS-like sequence [11,21] and also with integration of the marker within mitochondria that show this segregation. The high frequency of carbenicillin-resistant *E. coli* transformants make the former possibility more likely. Plasmid pWP8 transforms *U. violacea* at a rate 2–3 times that of pMP4-1, suggesting that pWP8 contains an ARS-like sequence. Mapping experiments with the transformed *U. violacea* are underway to see if the neomycin-resistance phenotype is linked to any *U. violacea* markers. These experiments should help to characterize further expression of a prokaryotic replicon in a higher fungus.

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REFERENCES

1 Wang, J., D.W. Holden, and S.A. Leong. 1988. Gene transfer system for the phytopathogenic fungus *Ustilago maydis*. Proc. Nat. Acad. Sci. U.S.A. 85: 865–869.

2 Garber, E.D. and A.W. Day. 1985. Genetic mapping of a phytopathogenic basidiomycete, *Ustilago violacea*. Bot. Gaz. 146: 449–459.

3 Bej, A.K. and M.H. Perlin. 1989. A high efficiency transformation system for the basidiomycete *Utilago violacea* employing hygromycin resistance and lithium-acetate treatment. Gene 80: 171–176.

4 Perlin, M.H. and S.A. Lerner. 1986. High-level amikacin resistance in *Escherichia coli* due to phosphorylation and impaired uptake. Antimicrob. Ag. Chemother. 29: 216–224.

5 Garber, E.D. and A.E. Owens. 1980. Genetics of *Ustilago violacea*, IV. Characterization of white and yellow strains by recombination and complementation. Bot. Gaz. 141: 79–84.

6 Robison, L.R., R. Seligsohn and S.A. Lerner. 1978. Simplified radioenzymatic assay for chloramphenicol. Antimicrob. Ag. Chemother 13: 25–29.

7 Sprengel, R., B. Reiss and H. Schaller. 1985. A new sensitive method for qualitative and quantitative assay of neomycin phosphotransferase in crude cell extracts. Nuleic. Acids Res. 13: 893–909.

8 Perlin, M.H. and S.A. Lerner. 1979. Amikacin resistance associated with a plasmid-borne aminoglycoside phosphotransferase in *Escherichia coli*. Antimicrob. Ag. Chemother. 16: 598–604.

9 Maniatis, T., E.F. Fritsch and Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratories, Cold Spring Harbor, NY.

10 Roughgarden, J. 1979. pp. 116–119 In: Theory of population genetics and evolutionary ecology: an introduction. Macmillan, NY.

11 Murray, A.W. and J.W. Szostak. 1983. Pedigree analysis of plasmid segragation in yeast. Cell 34: 961–970.

12 Dani, G.M. and V.A. Zakian. 1983. Mitotic and meiotic stability of linear plasmids in yeast. Proc. Nat. Acad. Sci. U.S.A. 80: 3406–3410.

13 Wills, J.W., B.A. Lasker, K. Sirotkin and W.S. Riggsby. 1984. Repetitive DNA of *Candida albicans*: nuclear and mitochondrial components. J. Bacteriol. 157: 918–924.

14 Cryer, D.R., R. Eccleshall and J. Marmur. 1975. Isolation of yeast DNA. Methods Cell Biol. 12: 39–44.

15 Garber, R.C. and O.C. Yoder. 1983. Isolation of DNA from filamentous fungi and separation into nuclear, mitochondrial, ribosomal, and plasmid components. Anal. Biochem. 135: 416–422.

16 Miller, J.H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

17 Jimenez, A. and J. Davies. 1980. Expression of a transposable antibiotic resistance element in *Saccharomyces cerevisiae*. Nature 287: 869–871.

18 Webster, T.D. and R.C. Dickson. 1983. Direct selection of *Saccharomyces cerevisiae* resistant to the antibiotic G418 following transformation with DNA vector carrying the kanamycin-resistance gene of Tn903. Gene 26: 243-252.

19 Spithill, T.W., P. Nagley and A.W. Linnane. 1979. Biogenesis of mitochondria 51. Mol. Gen. Gent. 173: 159–170.

20 Gale, E.F., E. Cundliffe, P.E. Reynolds, M.H. Richmond and M.J. Waring, (eds.). 1983. The molecular basis of antibiotic action, pp. 402–547, Wiley and Sons, NY.

21 Blackburn, E.H. 1985. Artificial chromosomes in yeast. Trends in Genetics. Jan. pp. 8–12.