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Abstract

A putative homolog of the *Sacharomyces cereviseae* *ADE6* and *Escherichia coli* *purL* genes is identified near a multigenic complex, which contains two genes, *sid1* and *sid2*, involved in a siderophore biosynthetic pathway in *Ustilago maydis*. The putative *ADE6* homolog was mutated by targeted gene disruption. The resulting mutant strains demonstrated a requirement for exogenous adenine, indicating that the *U. maydis ade6* homolog is required for purine biosynthesis.

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Disruption of a Yeast ADE6 Gene Homolog in *Ustilago maydis*

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A putative homolog of the *Saccharomyces cerevisiae* ADE6 and *Escherichia coli* purL genes is identified near a multigenic complex, which contains two genes, *sid1* and *sid2*, involved in a siderophore biosynthetic pathway in *Ustilago maydis*. The putative ADE6 homolog was mutated by targeted gene disruption. The resulting mutant strains demonstrated a requirement for exogenous adenine, indicating that the *U. maydis* ade6 homolog is required for purine biosynthesis.

Ustilago maydis is the causal agent of corn smut disease. Under conditions of iron stress, this fungus produces cyclic peptides, siderophores, for the purpose of iron acquisition (Leong and Winkelmann, 1998). The limits of this gene cluster were investigated by systematically analyzing the sequence of the flanking DNA. The *Ustilago* genomic sequence of the region downstream of *sid1* sequence showed a predicted 1402 amino acid polypeptide encoding a probable *ade6* gene (<http://mips.gsf.de/genre/proj/ustilago/singleGeneReport.html?entry=um05162>), and having 55.7% similarity to the translated *purL* gene of *E. coli*, and 54.2% similarity with the translated ADE6 gene of *Saccharomyces cerevisiae*. These genes encode formylglycineamide ribonucleotide synthetase, which catalyzes the fourth step in the purine biosynthetic pathway (Schendel *et al.*, 1989). Information contained in the *S. cerevisiae* Genbank sequence submission 557019 indicated that disruption of this gene leads to an adenine-requiring phenotype. To determine whether the predicted *ade6* gene is required for purine biosynthesis, the gene was disrupted by insertion of a cassette encoding hygromycin phosphotransferase.

Materials and Methods

The *HindIII-NruI* fragment containing the 5' region of the putative *ade6* gene was derived from an 8.2 kb *HindIII* fragment of pSid1, a cosmid clone that contains a *Sau3A* partial digest of a region of the genome of *Ustilago* strain 518 (Wang *et al.*, 1989), Table 1. The 8.2 kb *HindIII* fragment was initially cloned into pUC18 followed by deletion of *SmaI-NruI* and internal *NruI-NruI* fragments to yield the 2.5 kb cloned *HindIII-NruI* insert (Fig. 1). Plasmid DNA isolation from *E. coli* was performed using the alkaline lysis protocol (Maniatis *et al.*, 1982). *U. maydis* chromosomal DNA isolation was performed by the glass bead technique (Voisard *et al.*, 1993). Restriction enzyme digestions were carried out as suggested by the manufacturer (New England Biolabs). *E. coli* transformation was carried out using the calcium shock method (Maniatis *et al.*, 1982). *U. maydis* transformation was performed as described (Voisard *et al.*, 1993). Radiolabeling, DNA ligation and synthesis were carried out as using standard procedures (Maniatis *et al.*, 1982). Colony and Southern hybridizations were performed as described (Holden *et al.*, 1989). The translated sequences were aligned pairwise using the Lipman Pearson Method in Lasergene 7.1 (DNASTar, Madison) and by multiple alignment using Clustal W in Lasergene 7.1 (DNASTar, Madison) with the translated sequence of *ade6* generated in this study, a hypothetical Ade6 protein in the *Ustilago* genome (<http://mips.gsf.de/genre/proj/ustilago/singleGeneReport.html?entry=um05162>), *E. coli* PurL, and yeast Ade6.

Strain	Relevant Characteristics	Source
<i>U. maydis</i> #521 (FGSC 9914)	wild type <i>a1b1</i>	Robin Holliday, National Institute for Medical Research, Mill Hill, Great Britain
<i>U. maydis</i> #518 (FGSC 9914)	wild type <i>a2b2</i>	Robin Holliday, National Institute for Medical Research, Mill Hill, Great Britain

Table 1. Fungal Strains

Results

DNA sequence analysis of 2.5 kb *HindIII-NruI* fragment flanking the C- terminus of *sid1*

We sequenced the 2.5 kb *HindIII-NruI* fragment of *U. maydis* strain 518 that is immediately downstream of *sid1*. Open reading frame analysis revealed a single open reading frame encoding a protein of 547 amino acids within the 1870 bp of sequenced DNA of the 2.5 kb *HindIII-NruI* fragment and corresponding to a *BglIII-NruI* subregion. Gene identity was determined by Blastn and tBlastx analysis using data available in GenBank (www.ncbi.nlm.nih.gov). Analysis of the translated sequence against translated sequences in Genbank revealed 50 hits with significant sequence similarity (alignment score ≥ 200) for 56 accessions representing genome sequences from fungi and bacteria. Multiple alignment of the translated *Ustilago* gene with amino acid sequences of a putative Ade6 in the *Ustilago* strain 521 (Kamper *et al.*, 2006; <http://mips.gsf.de/genre/proj/ustilago/singleGeneReport.html?entry=um05162>), *E. coli* PurL and yeast Ade6 revealed significant similarity over the regions compared. The N-terminal region of 547 amino acids of the *Ustilago* strain 518 *ade6* showed complete identity with the predicted hypothetical protein for *ade6* for *Ustilago* strain 521 in the MIPS database. Sequence similarities of 54.3% with the translated *purL* gene of *E. coli* B and 48.6% with the translated *ADE6* gene of *S. cerevisiae* were observed over this same region (data not shown).

Disruption of putative *ade6* gene in *U. maydis*

Construction of plasmid pUC(2.5HindIII-NruI):hygB. *pUC(2.5HindIII-NruI)* DNA was digested at the single *MluI* site located near the middle of the 2.5 kb *HindIII-NruI* fragment encoding the 5' region of the putative *Ustilago ade6* gene (Fig. 1). The hygromycin resistance cassette was isolated as a *HindIII* fragment from pHLI (Wang *et al.*, 1989) after a double digestion with *HindIII* and *SspI*. The single-stranded ends of the 3.4 kb *HindIII* fragment encoding the hygromycin resistance gene were repaired with Klenow Polymerase and cloned into the *MluI* site of *pUC(2.5HindIII-NruI)* using T4 DNA ligase to produce the gene disruption construct.

Insert-containing clones were detected by hybridization of colony blots with a radioactively labeled hygromycin resistance gene probe. A restriction map of the cloned insert relative to the genomic region in which it is contained is shown in Figure 1.

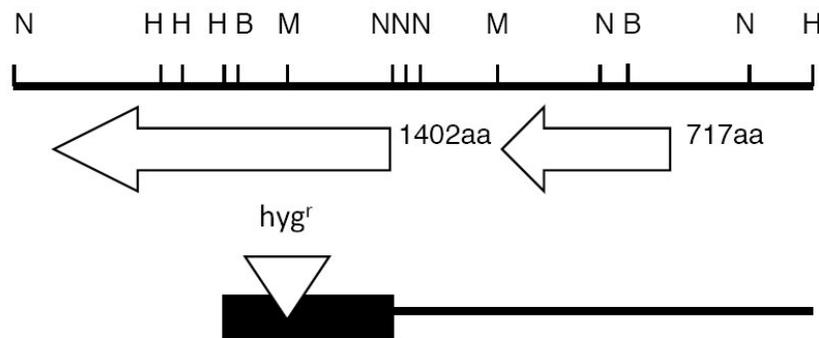


Figure 1. Partial restriction map of the *ade6-sid1* region of *U. maydis*. The *HindIII-NruI* fragment containing the hygromycin B resistance cassette inserted in the *MluI* site is shown at the bottom as a black box in relation to the 8.2 Kb *HindIII* fragment from which it was derived. *Ade6* encodes a 1402 amino acid polypeptide while *sid1* encodes a 717 amino acid polypeptide (Kamper *et al.*, 2007). Restriction sites shown are: B, *BglIII*; H, *HindIII*; M, *MluI*; and N, *NruI*.

A *HindIII/KpnI* digest was carried out to linearize the *ade6:hygB* DNA for single-step gene disruption of *U. maydis* (Fig. 1). The *KpnI* site is part of the pUC18 polylinker and is one bp distal to the *NruI:SmaI* fusion site of the *ade6:hygB* construct. pHLI, used as a control, was linearized with *HindIII*. The plasmids were transformed into *U. maydis* 518. Transformants were selected on hygromycin-containing PDA medium.

Complete Medium	Holliday, 1974
E-medium	Wang <i>et. al.</i> , 1989
Minimal Medium	Holliday, 1974
E-medium + adenine (10 mg/L)	Wang <i>et. al.</i> , 1989
Minimal + adenine (10 mg/L)	Wang <i>et. al.</i> , 1989

Table 2. Media

Sixty hygromycin resistant transformants were transferred onto complete medium + 300µg/ml hygromycin (Table 2) and subsequently to minimal medium, minimal medium + adenine, E medium, and E medium + adenine. The resulting growth patterns of eleven transformants were restricted on minimal and E medium, but were unrestricted on minimal + adenine, E medium + adenine, and complete medium. Southern hybridization analysis of the DNA of these 11 transformants using the 5' half of the *ade6* gene, and the hygromycin resistance gene, indicated that homologous integration had resulted in disruption of the putative *ade6* gene.

Segregation analysis was conducted on progeny obtained from three representative *ade6* disruption mutants of *Ustilago* after crossing with wild type strain 521 containing the opposite mating type. The growth of progeny on minimal medium with and without adenine is summarized in Table 3. Data from crosses with Mutants 4 and 5 are not consistent with a single gene segregating for adenine auxotrophy, while that for Mutant 6 does suggest a single gene is present ($p \gg 0.05$). The possibility exists that some colonies chosen from the primary plating of germinated basidiospores were not derived from single spores, since the number of adenine prototrophs exceeds that of adenine auxotrophs. In mixed colonies on minimal medium, adenine prototrophs would mask the poor growth of adenine auxotrophs. Mutant progeny may also have grown more slowly or shown poorer viability on the primary plating medium thus skewing the data in favor of prototrophs.

Cross	ade ⁺	ade ⁻	X ²	p
Mutant 4 X 521	39	11	15.7	<0.0001 ²
Mutant 5 X 521	33	17	5.1	0.024 ²
Mutant 6 X 521	27	23	0.32	0.57

Table 3. Segregation of adenine auxotrophy in crosses of disruption mutants with wild type.¹¹Degrees of freedom = 1²Significant

Discussion

A hypothetical protein showing significant identity to *purL* of *E. coli* and *ADE6* of *S. cerevisiae* was previously identified in the genome of *U. maydis* strain 521 (Kamper *et. al.*, 2006;

<http://mips.gsf.de/genre/proj/ustilago/singleGeneReport.html?entry=um05162>). In this study the N-terminal region of the putative *ade6* gene was sequenced from strain 518 and disrupted by single step gene disruption. The resulting transformants were incapable of growing on minimal and E media unless supplied with adenine as would be expected for disruption of the *U. maydis* formylglycineamide ribonucleotide synthetase gene. This gene marks one end of the gene cluster encoding siderophore biosynthetic functions *sid1* and *sid2*. Our data thus provide functional confirmation of the designation of the putative *ade6* gene at position 308279 to 304071 of contig 1.188 on Chromosome 4 of the genome sequence of *Ustilago maydis* strain 521 (Kamper *et. al.*, 2006; <http://mips.gsf.de/genre/proj/ustilago/singleGeneReport.html?entry=um05162>).

Resistance to the drugs hygromycin and phleomycin has been widely used in *Ustilago* for selection of transformants (e.g., An *et. al.*, 1997ab; Gold *et. al.*, 1994). The auxotrophic strains generated in this study can be used as recipients for *U. maydis* transformation by complementation. In addition, *ade6* mutants in combination with *ade1* or *ade2* mutants, that produce red pigments, can be used to design colony color screens for synthetic lethality and plasmid-generated mutations in a cloned gene (plasmid shuffle) (Sherman, 1998). The *ade6* mutation prevents the formation of the red phosphoribosylamino-imidazole pigment; therefore, loss of the *ade6*-complementing plasmid leads to white sectoring in the colony. This method allows for direct visual analysis of desired genetic events without the need to replica plate. With the availability of self-replicating plasmids these methods should be readily adapted to *Ustilago*.

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